Cyanobacteria as source of new antifungals

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Abbreviations

Abbreviations	Full name
ACN	Acetonitrile
AS	Anisaldehyd-sulphuric acid
BLAST	Basic Local Alignment Search Tool
COSY	Correlation spectroscopy
CFU	Colony forming unit
DAD	Diod Array Detector
DMSO	Dimethyl sulfoxide
Ē	Average
ESI MS	Electrospray ionization mass spectrometry
EtOH	Ethanol
GC/MS	Gas chromatography-mass spectrometry
GCQ	GCQ ion trap mass spectrometer
gHMBC	gradient-selected version of HMBC
HMBC	Heteronuclear multiple-bond correlation spectroscopy
HSQC	Heteronuclear single quantum correlation or Heteronuclear single quantum coherence
HPLC	High performance liquid chromatography
HMQC	Heteronuclear multiple-quantum correlation spectroscopy
IC_{50}	The half maximal inhibitory concentration
IMDM	Iscove's Modified Dulbecco's Medium
INT	Iodonitrotetrazolium Chloride
LOD	Limit of detection
LOQ	Limit of quantification
MEM	Mimimum Essential Medium Eagle
MeOH	Methanol
MIC	Minimal Inhibition Concentration
MS	Mass spectrometry
1D NMR	One dimensional nuclear magnetic resonance
2D NMR	Two dimensional nuclear magnetic resonance
NOESY	Nuclear Overhauser effect spectroscopy
OD	Optical Density
PBS	Phosphate buffered saline

PTFE	Polytetrafluorethylen
ROESY	Rotating frame nuclear overhauser effect spectroscopy
RP	Reversed phase
rRNA	Ribosomal ribonucleic acid
SPE	Solid phase extraction
SRC	Strange round cell
SS	Solvent system
STET Buffer	a mixture of NaCl, Tris buffer, EDTA and Triton X-100
TE Buffer	a mixture of Tris buffer and EDTA
TES	N-Tris-(hydroxymethyl)–methyl-2-amino-ethanesulfonic acid
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TOCSY	Total correlation spectroscopy
TYG	Tryptone yeast extract glucose medium
UV	Ultraviolet

Software

- FLUOstar Omega microplate readers (BMG LABTECH GmbH, Offenburg, Germany),
- MultiDoc-It Digital Imaging System (Ultra-Violet Products Ltd., Cambridge, GB),
- Doc-ItLS V. 6.1.1,
- Geminyx Version 1.91,
- LCsolution software, Shimadzu,
- GraphPad Prism 5.0,
- Microsoft Windows 7,
- Microsoft Office Excel 2010,
- Microsoft Office Powerpoint 2010,
- Microsoft Office Word 2010,
- ChemDraw Ultra 12.0,
- EndNote X6,
- Microscope Axioskop 2 *plus* (Carl Zeiss, Germany) coupled digital camera (AxioCam MRc camera, software Axiovision version 4),
- ImageJ software (Research Services Branch (RSB), National Institutes of Health (NIH)).

I. Introduction

I.1. Antifungal drugs in use, problems, and urgent need

Currently used antifungal drugs can be classified into five groups (Table I-1) based on their site of action in fungal cells (Bossche *et al.*, 1994; Andriole, 1999; Bossche *et al.*, 2003).

Since 1950s, griseofulvin, polyenes, and triazoles are still the most-commonly used classes of antifungal agents in therapy. Their antifungal activities target on ergosterol, a component of yeast and fungal cell membranes, which serves the same function as that of cholesterol in animal cells. This sterol does not occur in plant and animal cells. Therefore, it has become an attractive target for antifungal activity. The polyenes, amphotericin B and nystatin, selectively bind to ergosterol and disrupt the fungal plasma membrane (Brajtburg *et al.*, 1990). The azoles affect the biosynthesis of ergosterol and lead to an altered plasma membrane composition and affect membrane functions (Andriole, 1999). Griseofulvin interferes with the microtubule assembly process and thereby disrupts fungal cell division (Bowman *et al.*, 2006). Flucytosine works as an antifungal agent through its conversion to 5-fluorouracil within target cells. Fluorouracil then incorporates into RNA, causing premature chain termination, and it inhibits DNA synthesis through effects on thymidylate synthase. However, limitation of its spectrum on pathogen yeasts (restricted to *Candida* species and *C. neoformans*), as well as primary and secondary resistances has restricted the use of flucytosine in the clinic (Dixon *et al.*, 1996; Odds *et al.*, 2003).

Nevertheless, invasive fungal infections, particularly aspergillosis and fusariosis, in immunocompromised patients (cancer, transplants, premature infants, intensive therapy unit (ITU) patients, AIDS, etc.) have increased dramatically since 1990s (Andriole, 1999; Yamazaki *et al.*, 1999; Odds *et al.*, 2003; Nosanchuk, 2006). Treatments of these systemic fungal infections are restricted to few existing classes of agents, which are often associated with toxicity problems (Table I-2), administrative limitation, drug resistance, and limited spectrum of action (Table I-1) (Kontoyiannis *et al.*, 2002; Ashley *et al.*, 2006; Shao *et al.*, 2007; Shalini *et al.*, 2011). Therefore, systemic fungal infections continue to expand the urgent need for new antifungal agents, which should have a safety broad fungicidal spectrum of action, good pharmacokinetic properties and fewer dose-limiting side effects.

Table I-1. Important classes of antifungal agents based on mechanism of action

Targets		Chemical classes	Antifungal agents	Limitation
DNA and RNA synthesis (Pyrimidine analogues/ Thymidylate synthase inhibitors)		Pyrimidine	Flucytosine	Retricted to pathogenic yeasts, Drug resistance (Dixon *et al.*, 1996; Odds *et al.*, 2003)
Membrane barrier function		Polyenes	Amphotericin B	Serious nephrotocixity, drug interactions, resistance (Ryley *et al.*, 1981; Brajtburg *et al.*, 1990; Gallis *et al.*, 1990; Dixon *et al.*, 1996)
			Nystatin	Nephrotocixity, side effects (Ryley JF, 1981; Gallis, Drew and Pickard, 1990)
			Pimaricin (natamycin)	Topical use only (Dixon *et al.*, 1996)
Ergosterol synthesis	Squalene epoxidase	Allylamines	Naftifine, Terbinafine	Side effects (Ryder, 1987; Dixon *et al.*, 1996)
			Butenafine	(Iwatani *et al.*, 1993)
		Thiocarbamate	Tolnaftate	Limited to dermatophyte fungi. (Barrett-Bee *et al.*, 1986)
	14α-Demethylase	Azoles		Drug-drug interactions (Dvorak, 2011)
		Imidazoles	Bifonazole, Clotrimazole, Miconazole, Econazole, Ketoconazole	Hepatoxicity, drug interactions (Raab, 1978; Rotowa *et al.*, 1990; Yu *et al.*, 2005)
		Triazoles	Fluconazole, Itraconazole, Voriconazole Terconazole	Hepatoxicity, drug interactions (Gearhart, 1994; Yu *et al.*, 2005; Aperis *et al.*, 2006)
		Thiazole	Abafungin	(Borelli *et al.*, 2008; Kashyap *et al.*, 2012)
	Δ^{14} -Reductase $\Delta^{8} \rightarrow \Delta^{7}$ -Isomerase	Morpholines	Amorolfine	Only topical treatment (Odds *et al.*, 2003)
Mitosis inhibition			Griseofulvin	Limited to dermatophyte fungi. Hepatoxicity, drug interactions / adverse reactions (Williams, 1958; De Matteis, 1982; Okey, 1986; De Carli, 1988; Vanden Bossche, 1994)
1,3-β-D-Glucan synthesis		Echinocandins	Caspofungin, Micafungin, Anidulafungin	(Denning, 1997; Barrett, 2002; Saravolatz *et al.*, 2003)

Table I-2. Comparative toxicities of antifungal agents.

Type of toxicity	Antifungal agent										
	AmB	ABCD and ABLC	LAB	Flu	Itr	Vor	Pos	Ani.	Caspo.	Mica.	Flucy.
Hepatic	++	++	++	+	+	+	+	+	+	+	++
Nephrotic	++++	+++	++	-	-	-	-	-	-	-	-
Hematologic	+	+	+	NR	NR	NR	NR	NR	+	+	+++
Infusion-related	+++	+++	++	-	-	-	NA	+	+	+	NA
Electrolyte abnormalities[a]	+++	++	++	NR	+	+	NR	+	+	NR	+

Adapted from Ashley *et al*. (Ashley *et al.*, 2006). Plus signs indicate degree of toxicity: +, mild; ++, moderate; and +++, severe. AmB, amphotericin B; ABCD, amphotericin B colloidal dispersion; ABLC, amphotericin B liquid complex; LAB, liposomal amphotericin B; Flu, fluconazole; Itr, itraconazole; Vor, voriconazole; Pos, posaconazole; Ani., anidulafungin; Caspo., caspofungin; Mica., micafungin; Flucy., flucytosine; NA, data not available because of a lack of formulation; NR, not reported; [a] Includes hypokalemia and hypomagnesemia.

It is worth mentioning here that lipopeptides have become a potent group of antifungal agents. In a report of Newman published in 2012, among 29 antifungal drugs approved to market from 01/1981 to 12/2010, 19 drugs were azole-based compounds (Newman *et al.*, 2012) (Table I-3). Eight new agents, which reached the market just in the last ten years, include five new triazoles (eberconazole, fosfluconazole, luliconazole, posaconazole, and voriconazole) and three lipopeptides (caspofungin acetate, micafungin sodium, and anidulafungin) (Newman *et al.*, 2012; Singhal, 2013). These three lipopeptides are categorized as natural derivatives and belong to the group of echinocandins, a distinct and relative new class of antifungal agents that target the fungal cell wall (Cappelletty *et al.*, 2007). They inhibit the synthesis of 1,3-β-D-glucan, an essential homopolysaccharide in the wall of many fungal pathogens, which is not present in mammalian cells (Saravolatz *et al.*, 2003). The echinocandins represent the first novel target of antifungal drug discovery since 1980s in terms of clinically useful drugs (Odds *et al.*, 2003). They have emerged as a promising therapy for aspergillosis and candidiasis without cross-resistance to existing agents (Groll *et al.*, 2001). The lipopeptide caspofungin (1) is a semisynthetic derivative of pneumocandin B0 (2) - a fermentation product of the fungus *Glarea lozoyensis*. Caspofungin is the first of the echinocandins class which was approved in 2001 by the US Food and Drug Administration (FDA) for treatment of invasive aspergillosis in patients who are refractory to or intolerant of treatment using amphotericin B, lipid formulations of amphotericin B, and/or itraconazole (Groll *et al.*, 2001; Saravolatz *et al.*, 2003). Compared to amphotericin B, caspofungin seems to have a relatively low incidence of side effects (Groll *et al.*, 2001; Saravolatz *et al.*, 2003). Micafungin (3) received the final approval from the U.S. Food and Drug Administration in 2005, and is indicated for candidemia and candidosis treatment (Barrett, 2002; Jarvis *et al.*, 2004). A

clinical trial (number NCT00106288) published in 2007 concluded that micafungin was as effective as liposomal amphotericin B in the first-line treatment of candidemia and invasive candidosis (Kuse *et al.*, 2007). It also caused fewer adverse events than liposomal amphotericin B. Anidulafungin (4) is available for use since 2006 (Menichetti, 2009). Currently, all three agents are approved for the treatment of oesophageal candidiasis, candidaemia and other selected forms of invasive candidiasis including intra-abdominal abscesses and peritonitis (Kuti *et al.*, 2010; Chen *et al.*, 2011). Resistance to echinocandins is still rare and all agents are well tolerated, with similar adverse effect profiles and few drug interactions (Sucher *et al.*, 2009; Chen *et al.*, 2011). Overall, the three agents are relatively safe and effective agents for the treatment of *Candida* infections (Cappelletty *et al.*, 2007; Sucher *et al.*, 2009; Felder *et al.*, 2010; Chen *et al.*, 2011). Till now, the remained problem is echinocandins are expensive to use (Chen *et al.*, 2011).

Up till now, the prime requirement for new antifungal drug candidates is a broad antifungal spectrum of susceptible species, fungicidal activity both *in vitro* and *in vivo*, and fewer dose-limiting side effects (Andriole, 1999; Barrett, 2002; Odds *et al.*, 2003).

There are a number of new antifungal compounds are under study (Groll *et al.*, 1998; Andriole, 1999; Sangamwar *et al.*, 2008). Examples of antifungal agents with new mechanism of actions are sordarins and nikkomycins. Sordarins are a class of antifungal agents that formerly abandoned in the early 1970s and have received recent interest although not developed for clinical use (Andriole, 1999; Bueno *et al.*, 2002; Odds *et al.*, 2003; Liang, 2008). Sordarins (GM193663 (5), GM531920 (6)) inhibit fungal protein synthesis by blocking the function of fungal translation elongation factor 2 (EF2) (Bueno *et al.*, 2002; Liang, 2008). Nikkomycins (7) are nucleoside-peptide antibiotics produced by *Streptomyces* species. Their antifungal activities target the synthesis of chitin, one main component of the fungal cell wall found in most medically important fungi but mammalian cells (Li *et al.*, 1999). Besides, a new-generation of triazole antifungal drugs such as albaconazole (8), isavuconazole (9) and ravuconazole (10) are in Phase II and III trials (Girmenia *et al.*, 2011).

Table I-3. Antifungal Drugs from 01/01/1981 to 12/31/2010

(organized according to year introduced to the market)

No.	Generic name	Trade name	Year introduced	Source
1	anidulafungin	Eraxis	2006	ND
2	eberconazole	Ebernet	2005	S
3	luliconazole	Lulicon	2005	S
4	posaconazole	Noxafil	2005	S
5	fosfluconazole	Prodif	2003	S
6	micafungin sodium	Fungard	2002	ND
7	voriconazole	Vfend	2002	S
8	caspofungin acetate	Cancidas	2001	ND
9	liranaftate	Zefnart	2000	S/NM
10	interferon gamma-n1	OGamma100	1996	B
11	flutrimazole	Micetal	1995	S
12	lanoconazole	Astat	1994	S
13	neticonazole HCl	Atolant	1993	S
14	butenafine hydrochloride	Mentax	1992	S/NM
15	sertaconazole nitrate	Dermofix	1992	S
16	amorolfine hydrochloride	Loceryl	1991	S
17	terbinafine hydrochloride	Lamisil	1991	S/NM
18	fluconazole	Diflucan	1988	S
19	itraconazole	Sporanox	1988	S
20	fenticonazole nitrate	Lomexin	1987	S
21	butoconazole	Femstat	1986	S
22	cloconazole HCl	Pilzcin	1986	S
23	sulconazole nitrate	Exelderm	1985	S
24	naftifine HCl	Exoderil	1984	S
25	oxiconazole nitrate	Oceral	1983	S
26	terconazole	Gyno-Terazol	1983	S
27	tioconazole	Trosyl	1983	S
28	ciclopirox olamine	Loprox	1982	S
29	ketoconazole	Nizoral	1981	S

Adapted from the work of David J. Newman and Gordon M. Cragg (Newman *et al.*, 2012)

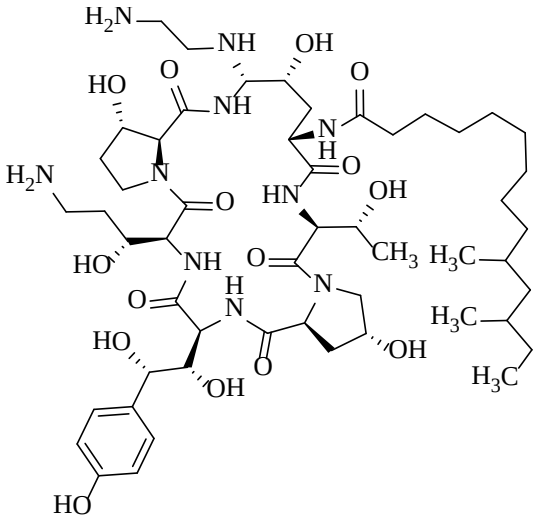
Major Categories of Sources:

"B" Biological; usually a large (>45 residues) peptide or protein either isolated from an organism/cell line or produced by biotechnological means in a surrogate host.

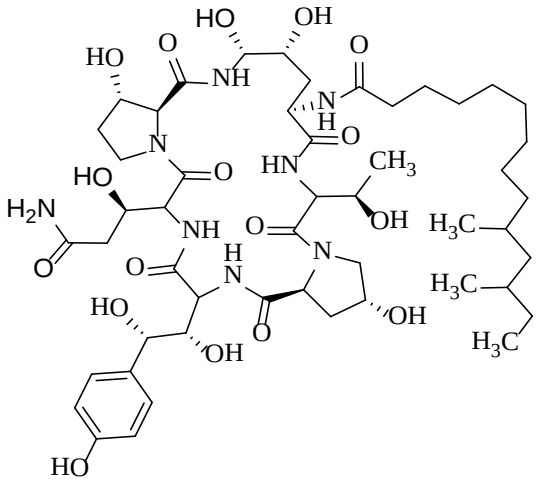
"ND" Derived from a natural product and is usually a semisynthetic modification.

"S" Totally synthetic drug, often found by random screening/modification of an existing agent.

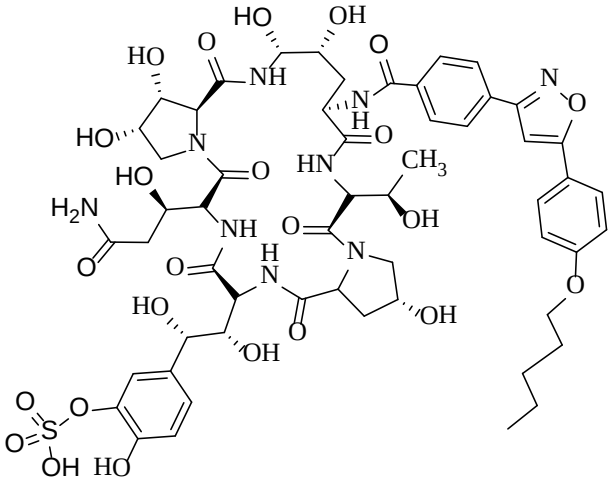
"NM" Natural product mimic.



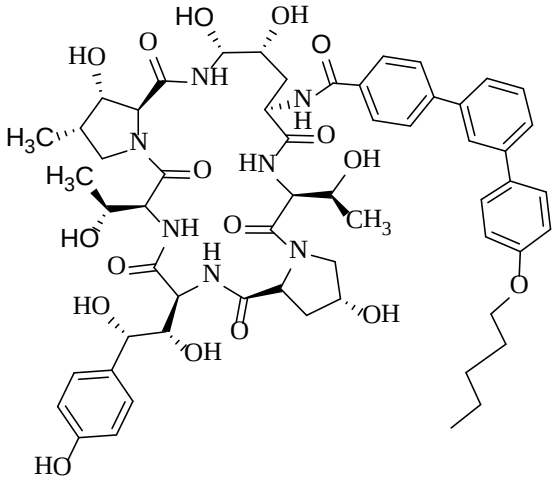
1 Caspofungin



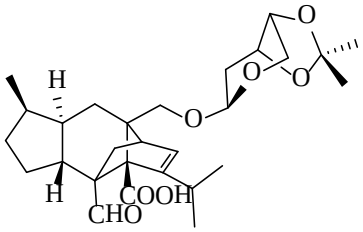
2 Pneumocandin B0



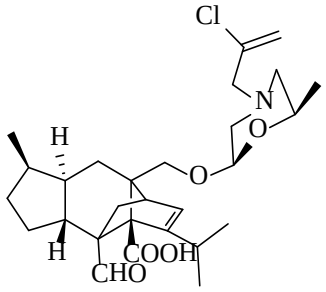
3 Micafungin



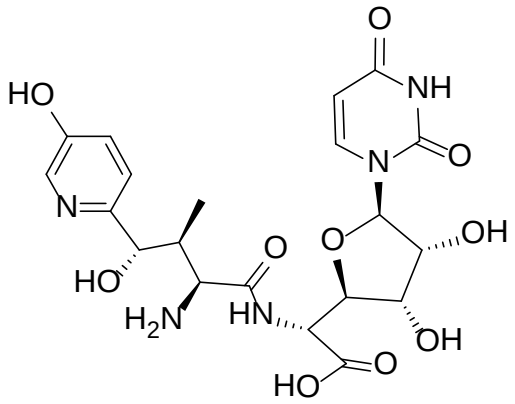
4 Anidulafungin



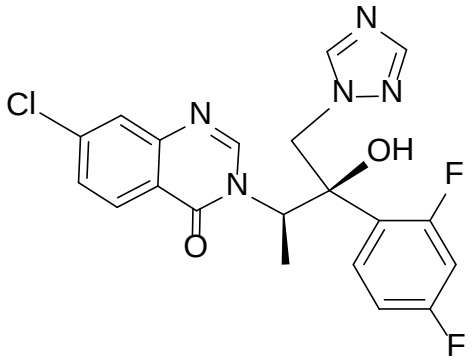
5 GM193663



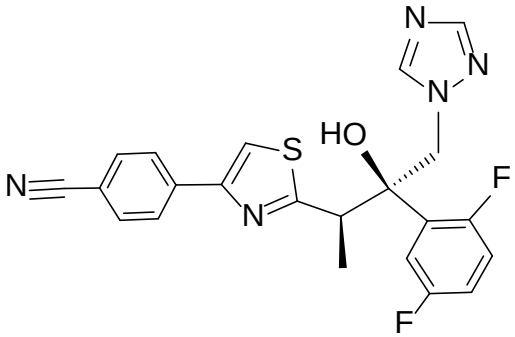
6 GM531920



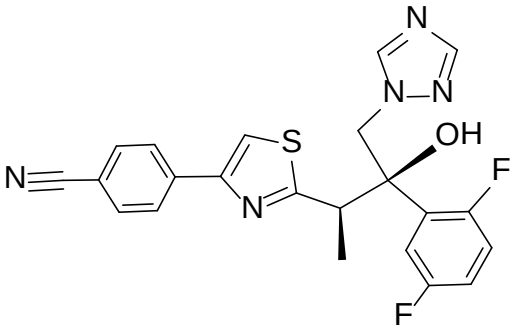
7 Nikkomycin Z



8 Albaconazole



9 Isavuconazole



10 Ravuconazole

It is no doubt that parallel with the increase of invasive fungal infections, the clinical need for antifungal agents have altered considerably and continuously since the specific antifungal agents have been discovered in 1950s (Odds *et al.*, 2003). However, the searching of effective antifungal agents for clinical use continues to be a major challenge because the antifungal compounds normally possess both antifungal activity and cytotoxicity (Andriole, 1999; Bueno *et al.*, 2002).

I.2. Physiology and taxonomic classification of cyanobacteria

Cyanobacteria (blue–green algae) are photoautotrophic, prokaryotic microorganisms distributed in diverse habitats, including also hot springs, Antarctic lakes and soils, and extreme euryhaline and eurythermal environments (Friedmann *et al.*, 1974; Colyer *et al.*, 2005; Tiwari *et al.*, 2005). Cyanobacteria lack cellulose in outer cell wall, do not reproduce sexually and have no membrane-bound nuclei or specialized organelles (Zaccaroni *et al.*, 2008). The main pigments of cyanobacteria are chlorophyll *a* and phycobiliproteins organized as phycobilisomes - light harvesting pigment complexes of phycobiliproteins - which are typical for cyanobacteria and responsible for the characteristic color of these organisms.

There are many different methods to classify cyanobacteria but the taxonomic identification itself mostly based on morphological features. According to the botanical nomenclature, assigned by Rabenhorst 1865, Bornet and Flahault 1886-1888, Gomont 1892, Geitler 1932, 1942, Desicachary 1959, Stamarch 1966, Bourrelly 1970 and Kondrateva 1975 (Geitler, 1932; Silva *et al.*, 1987), cyanobacteria are classified based on morphology, reproduction and physiology. From this system, 150 genera and about 2000 species are known. According to the bacteriological taxonomy, in which bacteria were assigned under the International Code of Bacteriological Nomenclature, Cyanophyceae are divided into 5 sections (Table I-4), namely Chroococcales, Pleurocapsales, Nostocales, Oscillatoriales, and Stigonematales (Stanier *et al.*, 1971; Stanier *et al.*, 1978; Rippka *et al.*, 1979; Castenholz, 1989a, b; Waterbury, 1989; Waterbury *et al.*, 1989; Waterbury, 2006). These two taxonomic systems were largely relied on morphological features.

The modern cyanobacterial classification established by Anagnostides and Komarek (Anagnostidis *et al.*, 1985, 1986, 1988, 1989, 1990) is based on morphological, physiological characteristics, and biochemical standards. In this system, cyanobacteria are also assigned under the International Code of Bacteriological Nomenclature.

Modern molecular genetic methods for taxonomic characterization are based on analogy of highly conserved regions in the 16S rRNA, 23S rRNA genes and other qualitative markers such as biochemical markers (cyanotoxins, oligopeptides, etc. (Pearson *et al.*, 2010)) or sequence of *hetR*, *rpoB*, *rbcLX*, *mcy*-genes, *gvp*-genes, *nif*-genes, etc. (Rajaniemi *et al.*, 2005; Tanabe *et al.*, 2007; Neilan *et al.*, 2008). These approaches have allowed determination of phylogenetic affiliations among cyanobacteria (Komárek, 2005, 2006; Neilan *et al.*, 2008; Pearson *et al.*, 2010).

According to the National Center for Biotechnology Information (NCBI), which provides unique taxonomic identifiers for all organisms (taxons) that are represented in the international nucleotide sequence database and in UniProtKB (The UniProt Knowledgebase), cyanobacteria were classified into seven orders (Table I-5).

Table I-4. The principal groups of cyanobacteria

(A comparison of modern and early systems of classification. Adapted from Singh *et al.* (2005)).

Section Rippka *et al.* (1979)	Basic morphology	Reproduction	Plane of division	Order (Family) Fritsch (1945)	Representative genera
I	Unicellular or colonial	Binary fission	Single	Chroococcales	*Gloeothece*, (*Anacystis*, *Agmenellum*) *Synechococcus*, *Gloeobacter*
			Two or more		*Chroococcus*, *Merismopedia, Microcystis*, *Gloeocapsa*, *Synechocystis*
		Budding		Chamaesiphonales	*Chamaesiphon*
II	Unicellular or colonial	Multiple fission			*Chroococcidiopsis, Dermocarpa*, *Dermocarpella*
				Pleurocapsales	*Hyella, Dermocarpella, Myxosarcina*, *Pleurocapsa* group, *Xenococcus*
III	Filamentous non-heterocystous	trichome fragmentation, normogonia	Single	Nostocales (Oscillatoriaceae)	*Lyngbya, Microcoleus*, *Oscillatoria*, *Phormidium, Plectonema, Pseudanabaena*, *Schizothrix, Spirulina*
IV	Filamentous heterocystous	Trichome breakage, hormogonia, akinetes	Single	(Nostocaceae)	*Anabaena, Anabaenopsis, Aphanizomenon, Cylindrospermum, Nodularia, Nostoc*
				(Rivulariaceae)	*Calothrix, Dichothrix, Gloeotrichia, Rivularia*
				(Scytonemataceae)	*Scytonema, Tolypothrix*
V	Branched filamentous, heterocystous	Trichome breakage, hormogonia, akinetes	Two or more	Stigonematales	*Chlorogloeopsis, Fischerella, Hapalosiphon, Mastigocoleus, Mastigocladus, Nostochopsis, Stigonema, Westiella*

Table I-5. Cyanobacterial taxonomic scheme according to NCBI Taxonomy Browser (August 31, 2009).

Order Family	Representative genera
Order Chroococcales	*Chamaesiphon, Chroococcus, Merismopedia, Microcystis, Gloeothece, Gloeocapsa, Synechococcus, Synechocystis,*
Order Gloeobacterales	*Gloeobacter*
Order Nostocales	
Family Microchaetaceae	*Coleodesmium, Fremyella, Hassallia, Microchaete, Petalonema, Rexia, Spiriresti, Tolypothrix*
Family Nostocaceae	*Anabaena, Anabaenopsis, Aphanizomenon, Cylindrospermum, Nodularia, Nostoc*
Family Rivulariaceae	*Calothrix, Gloeotrichia, Rivularia*
Family Scytonemataceae	*Brasilonema, Scytonema, Scytonematopsis*
Order Oscillatoriales	*Lyngbya, Microcoleus, Oscillatoria, Phormidium, Planktothrix, Plectonema, Pseudanabaena, Schizothrix, Spirulina*
Order Pleurocapsales	*Chroococcidiopsis, Dermocarpa, Dermocarpella, Myxosarcina, Pleurocapsa, Xenococcus*
Order Prochlorophytes	
Family Prochloraceae	*Prochloron*
Family Prochlorococcaceae	*Prochlorococcus*
Family Prochlorotrichaceae	*Prochlorothrix*
Oder Stigonematales	*Chlorogloeopsis, Fischerella, Hapalosiphon, Mastigocladopsis, Mastigocladus, Nostochopsis, Stigonema, Westiellopsis*

I.3. Cyanobacteria as source of novel natural products

I.3.1. Pharmaceutical compounds originated from cyanobacteria in clinical trial

A paper of Gerwick *et al.* (2012) highlighted recent trends in marine natural product research revealed that heterotrophic bacteria and cyanobacteria accounted for fully 80% of products which were approved as pharmaceutical agents or present in clinical trials. Besides, an analysis of 121 products that are available in biomedical research revealed that sponges, cyanobacteria and algae/plants (microalgae, macroalgae, and aquatic plants) are the sources of most of these products (Gerwick *et al.*, 2012) (Figure I-1).

In the last decades cyanobacteria have been recognized as sources of novel cytotoxic and antifungal metabolites (Sharma *et al.*, 2011). Some of these metabolites such as cryptophycin 52, soblidotin, synthadotin have potential for development of new pharmaceutical compounds (Liu *et al.*, 2010).

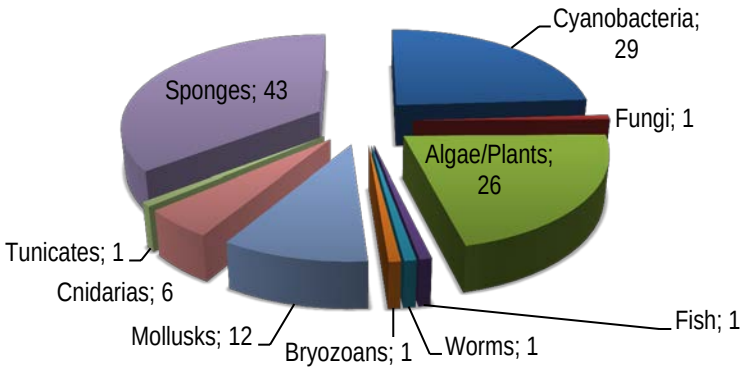
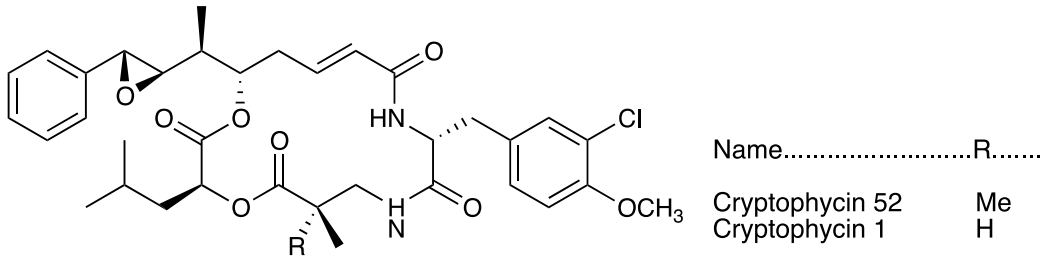
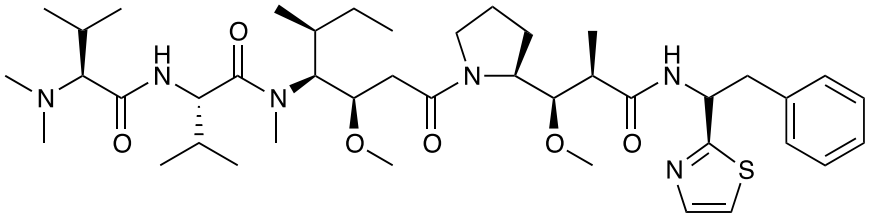


Figure I-1. The collected sources of marine natural products used as research biochemicals.

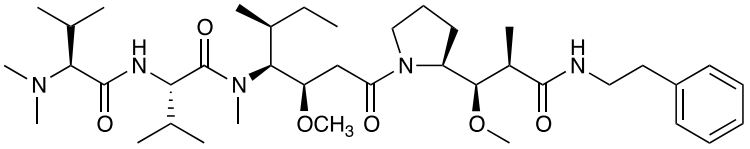
The synthetic analog cryptophycin 52 (11) of cryptophycin 1 (12), a cyclic depsipeptide previously isolated from *Nostoc* sp. ATCC 53789 as an antifungal agent (Trimurtulu *et al.*, 1994), progressed to Phase II of clinical trials for the treatment of patients with platinum-resistant ovarian cancer (Trimurtulu *et al.*, 1994; D'Agostino *et al.*, 2006). Dolastatin 10 (13), a peptide originally isolated from the Indian Ocean sea hare *Dolabella auricularia* (Pettit *et al.*, 1987) and later isolated from the marine cyanobacterium *Symploca* sp. VP642 (Luesch *et al.*, 2001), was an early promising antitumor agent. Nevertheless, it failed to demonstrate significant clinical activity in phase II of clinical trials (Vaishampayan *et al.*, 2000). Another synthetic analog of dolastatin 10, such as soblidotin (or TZT-1027, auristatin PE) (14), displayed promising activity against human colon adenocarcinomas but was unsuccessful due to the lack of efficacy and induced peripheral neuropathy in phase II clinical trials (Gerwick *et al.*, 2012). However, monomethyl auristatin E (15)- a synthetic analog of dolastatin 10 which conjugated to the anti-CD30 monoclonal antibody cAC10 resulted in brentuximab vendotin (Francisco *et al.*, 2003) gained FDA approval for use in anaplastic large cell lymphoma and Hodgkin's lymphoma in August 2011 (Gerwick *et al.*, 2012). Synthadotin (Tasidotin or ILX-651) (16), derived from dolastatin 15 (17) showed promising results in phase II clinical trials of inoperable, locally advanced or metastatic melanoma (Chetsumon *et al.*, 1994; Ebbinghaus *et al.*, 2004).



11-12 (Liu *et al.*, 2010)

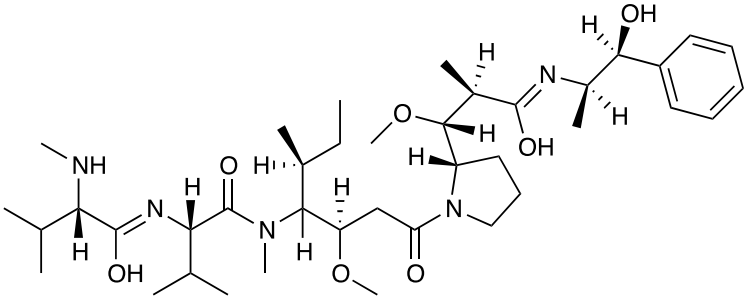


13 Dolastatin 10

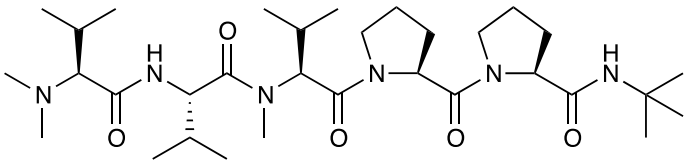


Soblidotin (TZT-1027, Auristatin PE)

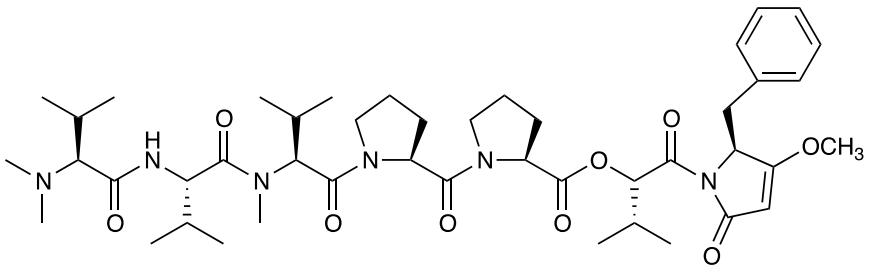
14



15 Monomethyl auristatin E



16 Synthadotin or Tasidotin ILX-651



17 Dolastatin 15

I.3.2. Nitrogen-containing secondary metabolites from cyanobacteria

More than 4000 strains of cyanobacteria have been studied to date, with more than 1000 secondary metabolites described (Gademann *et al.*, 2008; Kim *et al.*, 2012). A literature study indicated that approximately 68% of natural products derived from marine cyanobacteria contain nitrogen (Burja *et al.*, 2001). Up to 2007, over 300 nitrogen-containing secondary metabolites from marine cyanobacteria

have been reported in literature (Tan, 2007). In fact, peptides or peptide-containing substructures form a major group of cyanobacterial secondary metabolites (Moore, 1996; Namikoshi *et al.*, 1996; Burja *et al.*, 2001; Singh *et al.*, 2005; Liu *et al.*, 2010). Further analysis of Burja of 424 marine cyanobacterial natural products showed that 40.2% are lipopeptides (cyclic or linear), 5.6% are pure amino acid compositions, 4.2% are fatty acids, 4.2% are macrolides and 9.4% are amides (Burja *et al.*, 2001). The structures and bioactivities of 50 new *Lyngbya* peptides were reported from 2006 to 2010 (Liu *et al.*, 2010).

Cyanobacterial lipopeptides have been identified as interesting biochemically active compounds. Approximately 85% of them are bioactive, including cytotoxic (41%), antitumor (13%), antibiotic (12%), enzyme inhibitor (8%), antiviral (4%) and antifungal activities (4%) (Burja *et al.*, 2001) (Figure I-2). The remaining activity (18%) covers tumor promoting, herbicide, antimycotic, antimitotic, antimalarial, antimicroalgal, cell-differentiation promoting activity, cardioactivity and UV absorbing activity useable as sunscreens (Burja *et al.*, 2001).

From 2001 to 2006, 128 cyanobacterial alkaloids were published with a wide structural diversity and variety of biological actions such as antifungal activity, cytotoxicity, sodium channel modulation, and inhibition of proteases (Tan, 2007; Harvey, 2008; Vasas *et al.*, 2010). Majority of them were originated from the filamentous genera *Lyngbya*, *Oscillatoria*, and *Symploca* (Williams *et al.*, 2001, 2004; Osswald *et al.*, 2007; Grindberg *et al.*, 2008).

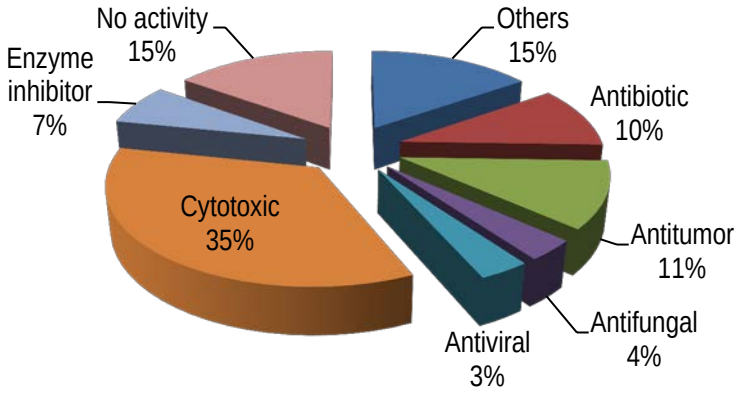


Figure I-2. Biochemical activity of lipopeptides isolated from cyanobacteria. (Burja *et al.*, 2001)

Some cyanobacteria have been used as human food for many years because of their high protein content (35–65%) and nutritional value (Venkataraman, 1997; Belay, 2002; Medina-Jaritz *et al.*, 2011). Among them, *Spirulina* (now *Arthrospira*) is the well-known example.

Moreover, cyanobacteria are known for the production of toxins associated with water blooms. About 40 genera of cyanobacterial species that are responsible for the production of freshwater and marine cyanobacterial toxins have been found (Grindberg *et al.*, 2008). These toxins, namely cyanotoxins, can be grouped according to their toxic mechanism in vertebrates as hepatotoxins, neurotoxins, irritants and dermatotoxins, and general cytotoxins. They fall into three broad groups of chemical structures: cyclic peptides, alkaloids and lipopolysaccharides (Jones *et al.*, 1999; Stewart *et al.*, 2006). The best known cyanotoxins are microcystins and nodularins, which are potent inhibitors of protein phosphatases.

Together with those toxins, during the last few decades hundreds of cyanobacterial secondary metabolites were reported (Carmichael, 1992; Patterson *et al.*, 1994; Harada, 2004; Ferreira, 2006; Newman *et al.*, 2012). These metabolites possess different chemical structures such as fatty acids (Wada *et al.*, 1993 ; Mundt *et al.*, 2003), phenolics (Pedersén *et al.*, 1973; Cano *et al.*, 1990), terpenoids (Jaki *et al.*, 1999), N-glycosides (Bonjouklian *et al.*, 1991), lipopeptides (MacMillan *et al.*, 2002; Neuhof *et al.*, 2005; Neuhof *et al.*, 2006), linear and cyclic peptides (Pergament *et al.*, 1994; Burja *et al.*, 2001; Tan, 2007; Zainuddin *et al.*, 2009) and alkaloids (Moore *et al.*, 1987b; Bonjouklian *et al.*, 1991; Raveh *et al.*, 2007; Mo *et al.*, 2009). In addition, they exhibit a diverse spectrum of biological activities including antibacterial (Soltani *et al.*, 2005; Svircev *et al.*, 2008), algicidal (Dale *et al.*, 2003), antifungal (Frankmölle *et al.*, 1992a; Jaki *et al.*, 2001; Zorica Svircev, 2008) antiviral (Zainuddin *et al.*, 2002), anticancer (Gerwick *et al.*, 1994), cytotoxic (Thornburg *et al.*, 2011), and enzyme inhibiting activities (Cannell *et al.*, 1988a; Ishida *et al.*, 1995; Shin *et al.*, 1996). Therefore, cyanobacteria are considered as a source of potential pharmaceutical substances (Borowitzka, 1995; Newman *et al.*, 2012) including antifungal compounds.

I.3.3. Antifungal metabolites from cyanobacteria

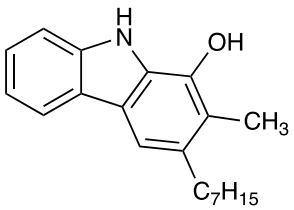
Several extracts of cyanobacteria belong to *Oscillatoriales, Nostocales*, and *Stigonematales* have shown antifungal activity in *in-vitro* test systems (Namikoshi *et al.*, 1996; Burja *et al.*, 2001; Ghasemi *et al.*, 2003; Bhadury *et al.*, 2004; Singh *et al.*, 2005; Soltani *et al.*, 2005; Abed *et al.*, 2009). Antifungal compounds isolated from these extracts are presented in Table I-6.

Table I-6. Selected antifungal compounds of cyanobacteria and representative producing species.

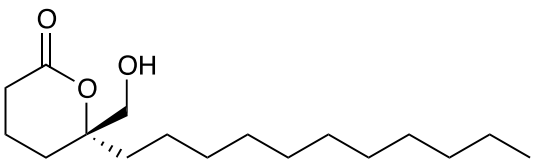
(Namikoshi *et al.*, 1996; Burja *et al.*, 2001; Singh *et al.*, 2005; Abed *et al.*, 2009)

Source	Compound	Activity	Class of compound	References
Order Chroococcales: no report until now				
Order Pleurocapsales				
Hyella caespitosa	Carazostatin (18)	antifungal	Alkaloid	(Cardellina II *et al.*, 1979)
Order Oscillatoriales				
Lyngbya majuscula	Tanikolide (19)	antifungal, cytotoxic	Lactone	(Singh *et al.*, 1999)
Lyngbya majuscula	Lyngbyabellin A, B (20, 21)	antifungal, cytotoxic	Cyclic depsipeptide	(Milligan *et al.*, 2000)
Lyngbya majuscula	Hectochlorin (22)	antifungal, cytotoxic	Cyclic depsipeptide	(Marquez *et al.*, 2002)
Lyngbya confervoides	Lobocyclamides A – C (23 - 25)	antifungal	Cyclic lipopeptide	(MacMillan *et al.*, 2002)
Schizothrix sp.	Schizotrin A (29)	antibacterial, antifungal	Cyclic peptide	(Pergament *et al.*, 1994)
Order Nostocales				
Anabaena laxa	Laxaphycins A and B (26, 27)	synergistically antifungal, cytotoxic	Cyclic lipopeptide	(Frankmölle *et al.*, 1992a; Frankmölle *et al.*, 1992b)
Hormothamnion enteromorphoides	Hormothamnins (28)	antifungal, cytotoxic	Cyclic peptide	(Gerwick *et al.*, 1989; Gerwick *et al.*, 1992)
Calothrix fusca	Calophycin (30)	antifungal	Cyclic peptide	(Moon *et al.*, 1992)
Nostoc commune	Nostofungicidine (31)	antifungal	Cyclic lipopeptide	(Kajiyama *et al.*, 1998)
Scytonema pseudohofmanni, *Scytonema mirabile, Scytonema burmanicum, Scytonema ocellatum,*	Scytophycins (32, 33)	cytotoxic, antifungal	Scytophycin-type macrolide	(Moore *et al.*, 1986; Stewart *et al.*, 1988) (Ishibashi, 1986; Carmeli *et al.*,

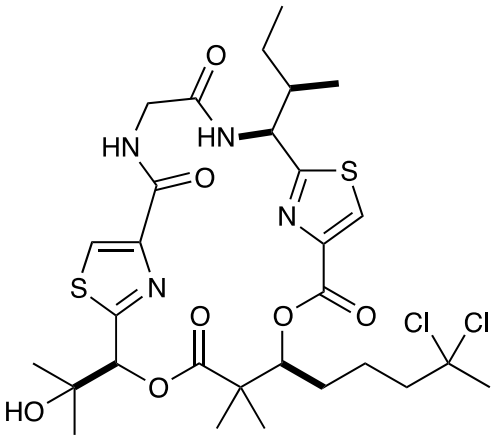
Cylindrospermum muscicola				1990a; Jung *et al.*, 1991; Smith *et al.*, 1993)
Tolypothrix conglutinate, *Scytonema mirabile*, *Scytonema ocellatum*, *Scytonema burmanicum*	Tolytoxin (34)	cytotoxic, antifungal	Nucleoside	(Moore *et al.*, 1986; Stewart *et al.*, 1988; Carmeli *et al.*, 1990b)
Tolypothrix byssoidea	Tubercidin (35)	cytotoxic, antifungal	Nucleoside	(Moore *et al.*, 1986; Stewart *et al.*, 1988)
Tolypothrix tenuis	Toyocamycin (36)	antifungal	Nucleoside	(Moore, 1982)
Tolypothrix tjipanasensis	Tjipanazoles (37)	antifungal	Alkaloid	(Bonjouklian *et al.*, 1991)
Tolypothrix sp.	Hassallidin A and B (38, 39)	antifungal	Cyclic peptides	(Neuhof *et al.*, 2005; Neuhof *et al.*, 2006)
Order Stigonematales				
Fischerella muscicola	Fischerellin (40)	antifungal, herbicidal	Lipopeptide	(Srivastava *et al.*, 1999)
Fischerella ambigua	Tjipanazole D	antifungal	Indole alkaloid	(El Omari, 2011)
Hapalosiphon fontinalis	Anhydrohapalox-indole (41), fontonamide (42),	antibiotic, antifungal	Indole alkaloids	(Moore *et al.*, 1987b)
	Hapalindoles (43)	antibiotic, antifungal	Indole alkaloids	(Moore *et al.*, 1987a)



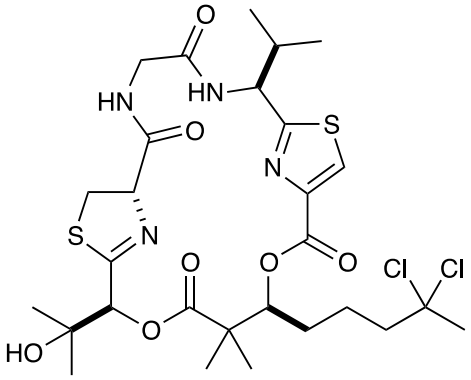
18 Carazostatin



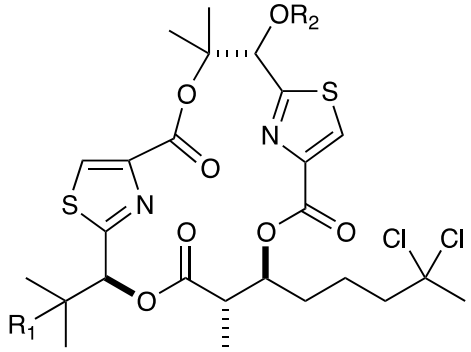
19 Tanikolide



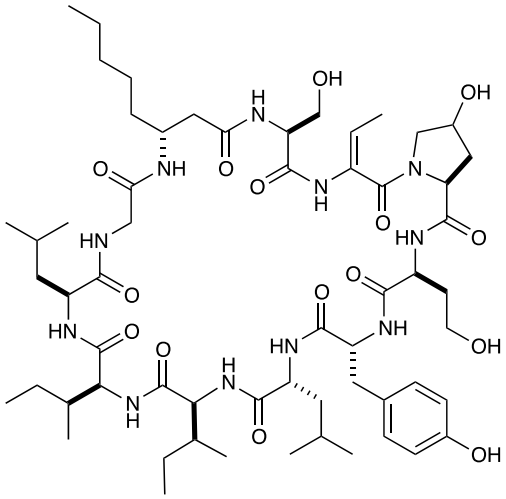
20 Lyngbyabellin A



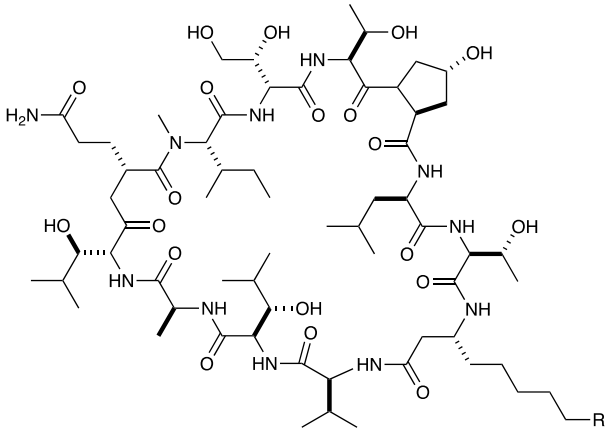
21 Lyngbyabellin B



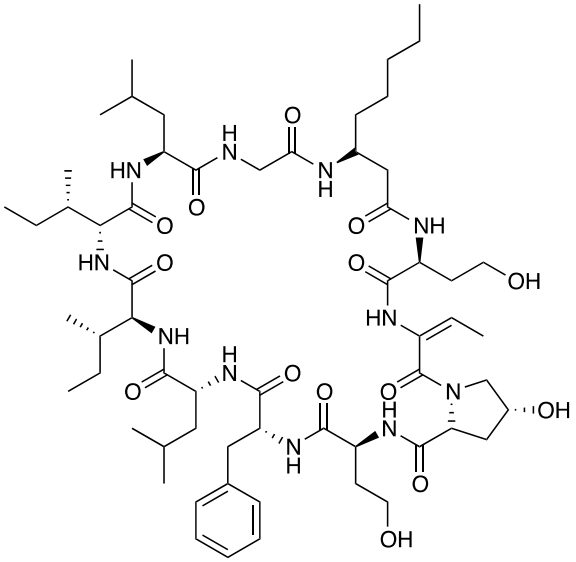
22 Hectochlorin R1 = OH, R2 = COCH3



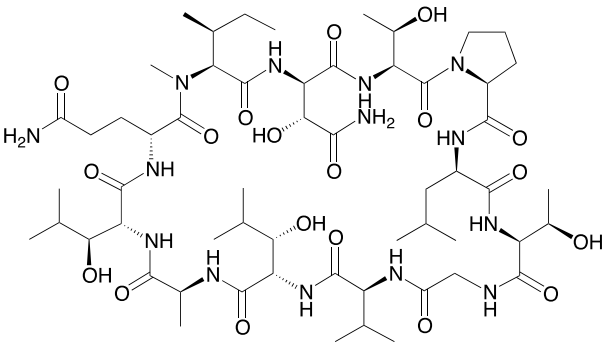
23 Lobocyclamide A



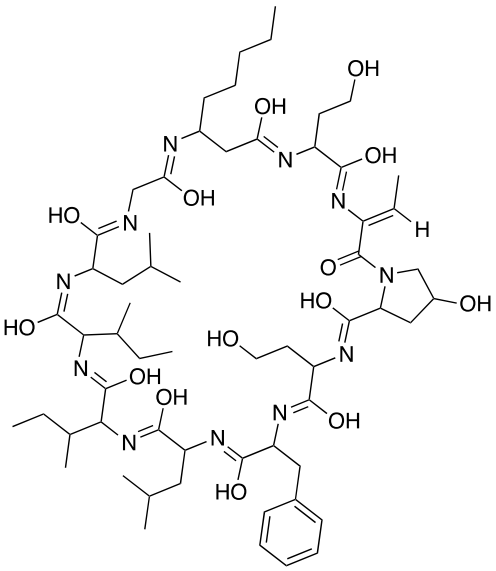
24 Lobocyclamide B R = CH_2CH_3
25 Lobocyclamide C R = H



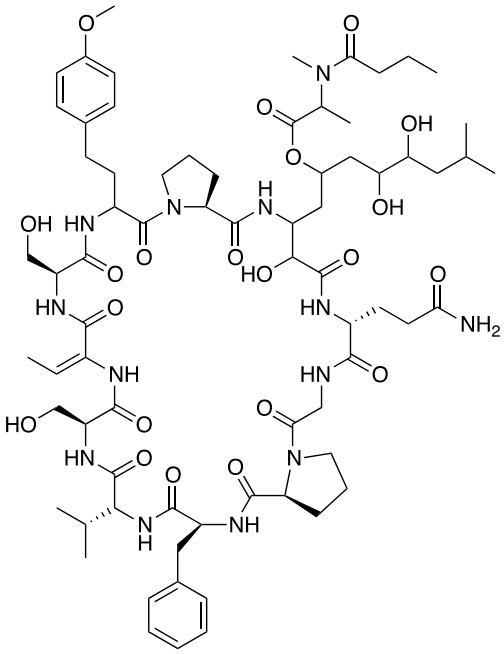
26 Laxaphycin A



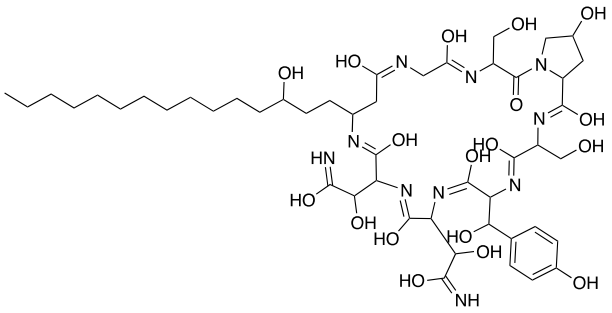
27 Laxaphycin B



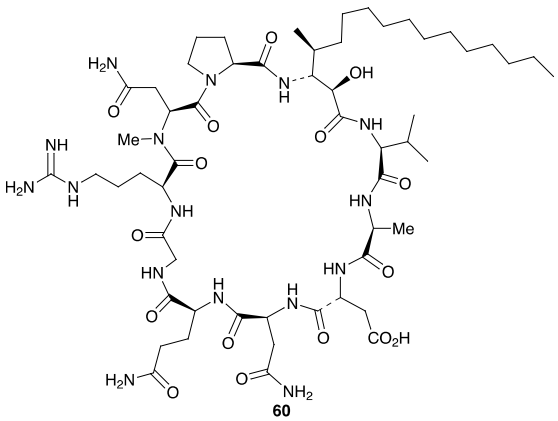
28 Hormothamnin A



29 Schizotrin A

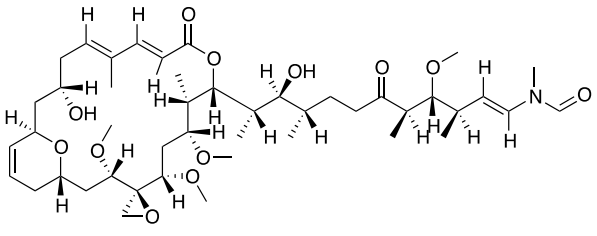


31 Nostofungicidine

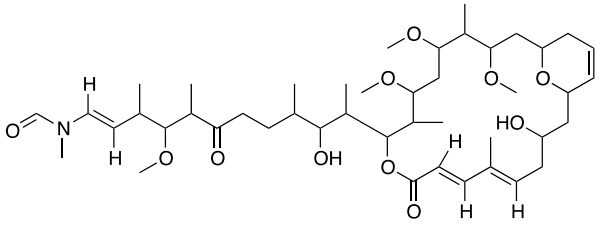


30 Calophycin

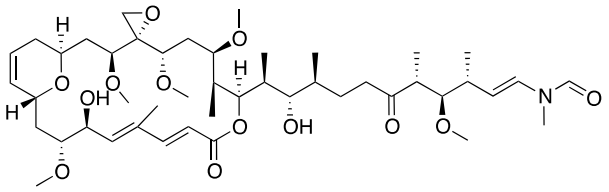
(*Nat. Prod. Rep.*, 1999, 16, 339–365)



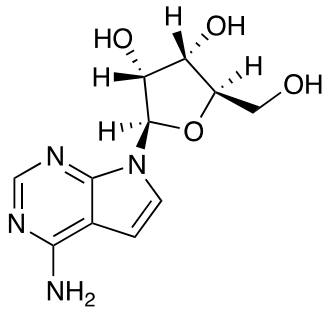
32 Scytophycin B



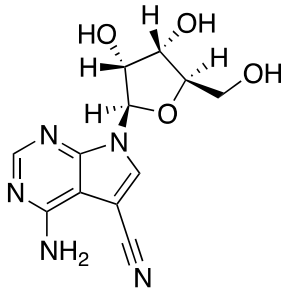
33 Scytophycin C



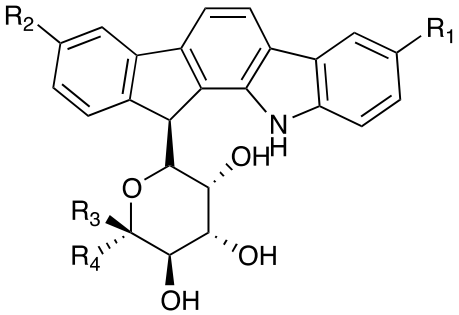
34 Tolytoxin



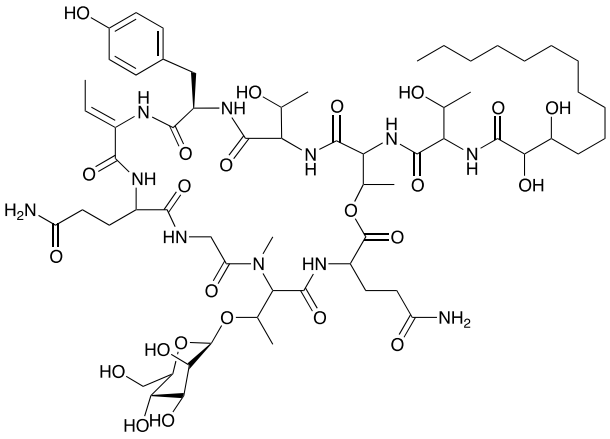
35 Tubercidin



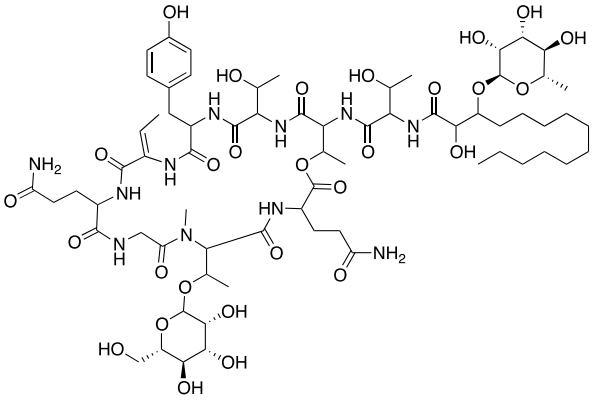
36 Toyocamycin



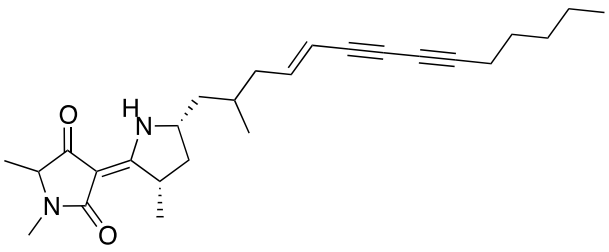
37 Tjipanazoles



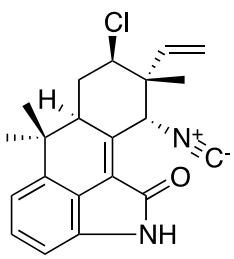
38 Hassallidin A



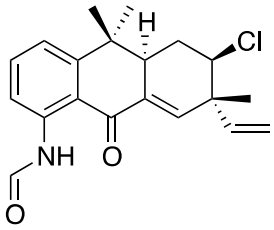
39 Hassallidin B



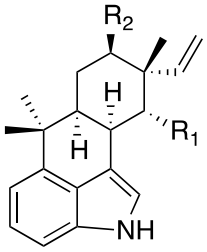
40 Fischerellin



41 Anhydrohapaloxindole



42 Fontonamide



43 Hapalindoles

Aim of work

A large number of reports about novel bioactive compounds isolated from cyanobacteria during the last few decades state that cyanobacteria are identified as a source of potential drugs. Due to increasing fungal infections and limitations of available drugs such as drug safety, resistance, drug–drug interactions, narrow spectrum of activity and effectiveness the discovery of novel antifungals with a safety broad fungicidal spectrum of action, good pharmacokinetic properties and fewer dose-limiting side effects is an important task for drug discovery.

In our continuing efforts to discover novel and biological active metabolites cyanobacterial strains isolated from water and soil samples, collected in North Vietnam near Hanoi, from the Baltic Sea near Rügen and Hiddensee and from ponds and water reservoirs in the North of Germany were investigated with the following aims:

1. Identification of extracts with specific antifungal activity by *in vitro* screening for antifungal activity followed by antibacterial screening of the intra- and extracellular crude extracts prepared with solvents with different polarity;

2. Isolation, identification and structural elucidation of active compounds from extracts exhibiting potent antifungal activity by bioassay-guided isolation to discover novel structures;

3. Culture optimization of selected strains which possess strong and specific antifungal activity to enhance yield of biomass and active compound production;

4. Taxonomic classification of cyanobacterial strains producing novel antifungal compounds.

II. Materials and methods

II.1. Cultivation of cyanobacteria

II.1.1. Stock cultures

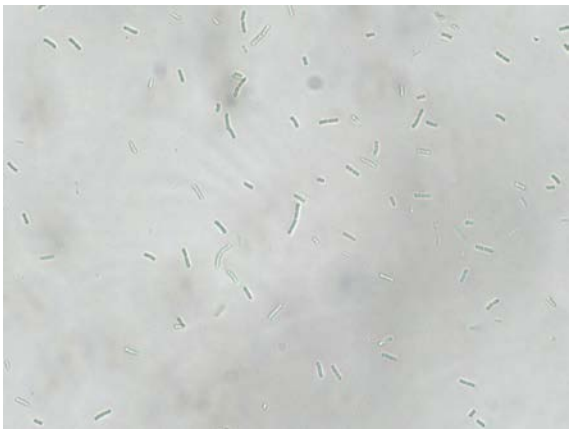
29 cyanobacteria, including 23 Vietnamese and 6 German strains, used in the antimicrobial screening are listed in Table II-1. The Vietnamese strains, were isolated from rice fields and shallows in Northern of Vietnam (Thanhhoa, Thaibinh, Namdinh and Hanoi). These strains were maintained in the culture collection of the Department of Pharmaceutical Biology, Institute of Pharmacy, Ernst-Moritz-Arndt University Greifswald, Germany.

Table II-1. Investigated cyanobacteria

No	Strain	Origin and Order	Medium	Morphology
Cultivated strains				
1.	AS	Well water sample/München Nostocales	BG 11	*Anabaena solitaria*. Filaments are not long, no branches; spherical to cylindrical cells; heterocytes and akinetes are spherical.
2.	154	Greifswald Oscillatoriales	BG 11	*Pseudanabaena catenata*. Short filaments, no branches; cells are rectangular, always longer than wide; no heterocyst.
3.	1611	Garden pond near Cambridge Nostocales	BG 11	*Anabaena cylindrica*. Thin filament contains cylindrical cells, no branches; heterocysts and akinetes are terminal or intercalar in the filament.
4.	CAVN 3	Nostocales	MBL	Filamentous; subspherical cells; the curved filaments are constricted at each cross-wall, the trichome looks like as string of beads; no heterocysts in filament; no branches.
5.	CAVN 4	Nostocales	BG 11	Single filament contains cylindrical cells; trichome looks like as string of beads; no heterocysts; no branches; akinetes are intercalar in the filament.
6.	CAVN 5	Nostocales	BG 11	Filament contains subspherical and cylindrical cells; no heterocysts in filament; no branches.
7.	CAVN 7	Nostocales	BG 11	Cells are square or long rectangular; the curved filaments are constricted at each cross-wall, so that the trichome looks like as string of beads; no heterocysts in filament; no branches.
8.	CAVN 8	Nostocales	BG 11	Filament contains cylindrical cells; trichome looks like as string of beads; no heterocysts; akinetes are terminal or intercalar in the filament.

9.	CAVN 12	Nostocales	BG 11	Long, thin filaments with oblong rectangular cells; no heterocysts; akinetes in long chains.
10.	CAVN 14	Oscillatoriales	BG 11	*Limnothrix* sp. (Fricke, 2006); long, thin filaments with rectangular cells; heterocysts, akinetes and branches are absent.
11.	CAVN 15	Nostocales	BG 11	Filamentous, cylindrical cells; akinetes are terminal or intercalar in the filament; no branches; no heterocysts.
12.	CAVN 17	Nostocales	BG 11	Filamentous, cells nearly spherical; the curved filaments are constricted at each cross-wall, the trichome looks like as string of beads; no heterocysts in filament; no branches; akinetes are long cylindrical.
13.	CAVN 20	Oscillatoriales	BG 11	Long trichomes without any branching, not constricted at the crosswalls; cells discoid, without heterocysts.
14.	TVN40	Nostocales	BG 11	*Nostoc* sp. Long trichomes constricted at the crosswalls, look like string of beads, cells barrel-shaped, mostly longer than broad; no heterocysts in filament; no branches.
15.	SRC	unidentified	BG 11+ Cobalt	Unicells or a colony of spherical cells covered with a distinct sheath.
16.	Bio 33	The Baltic Sea Grambow/Rügen Nostocales	BG 11 + 0.5% NaCl	*Anabaena cylindrica*. Single filament contains cylindrical cells. Heterocysts and akinetes appear intercalar in the filament.
Biomasses of the following strains were screened (not cultivated by the author)				
17.	CAVN 1	Nostocales	BG 11	*Nostoc entophytum*
18.	CAVN 2	Nostocales	BG 11	*Nostoc* sp.
19.	CAVN 10	Nostocales	BG 11	*Nostoc* sp.
20.	CAVN 11	Nostocales	BG 11	*Nostoc* sp.
21.	CAVN 16	Stigonematales	BG 11	*Fischerella musicola*
22.	TVN7	Nostocales	BG 11	*Nostoc spongiaeforme*
23.	TVN9	Nostocales	BG 11	*Nostoc* sp.
24.	TVN14	Nostocales	BG 11	*Nostoc coeruleum*
25.	TVN16	Oscillatoriales	BG 11	*Oscillatoria* sp.
26.	TVN201	Nostocales	BG 11	*Calothrix marchica*
27.	TVN202	Nostocales	BG 11	*Calothrix elenkinii*
28.	233	Meinigen bridge Oscillatoriales	BG 11	*Pseudanabaena catenata*
29.	1	The Baltic Sea/Hiddensee Nostocales	BG 11	*Anabaena variabilis*

AS (400x)



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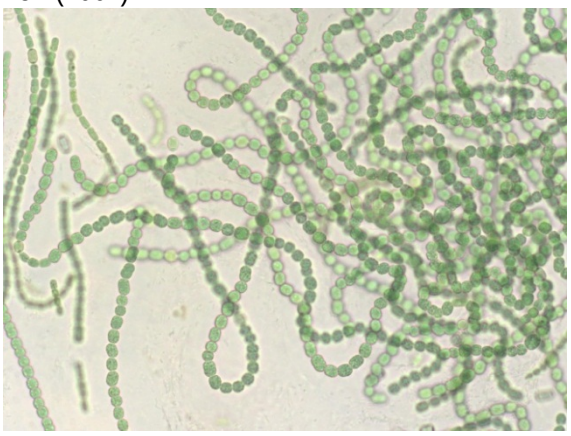
1611 (400x)



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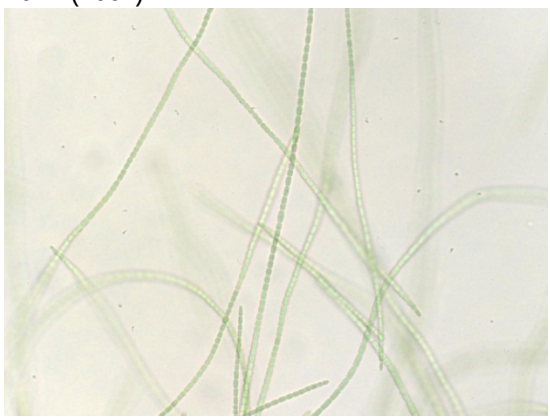

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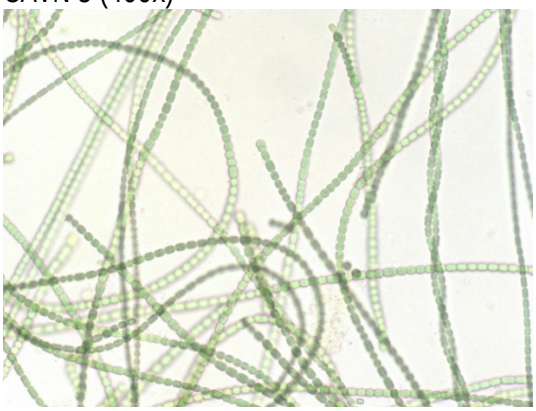
CAVN 5 (400x)

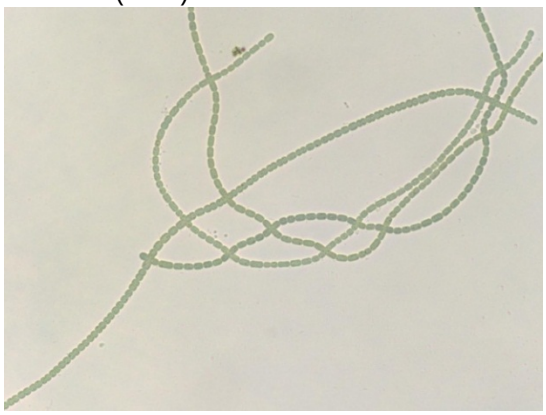
CAVN 7 (400x)



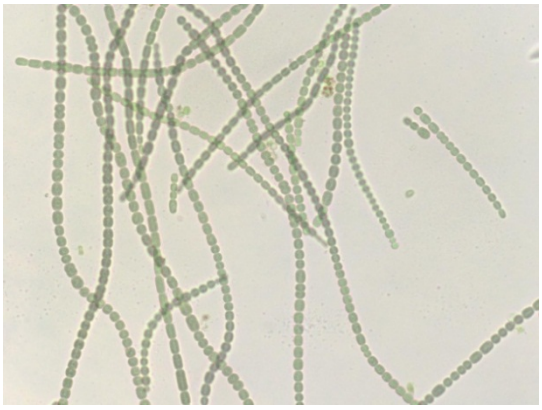
CAVN 8 (400x)

CAVN 12 (400x)

CAVN 14 (400x)



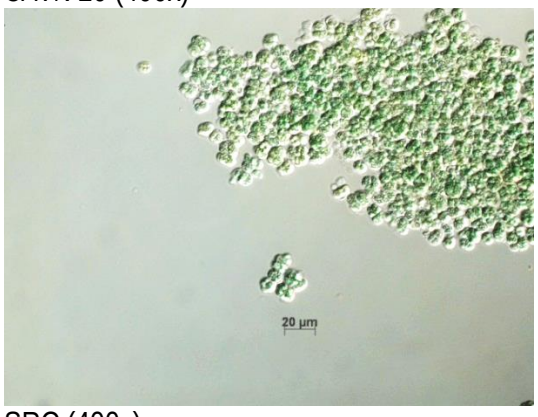
CAVN 15 (400x)



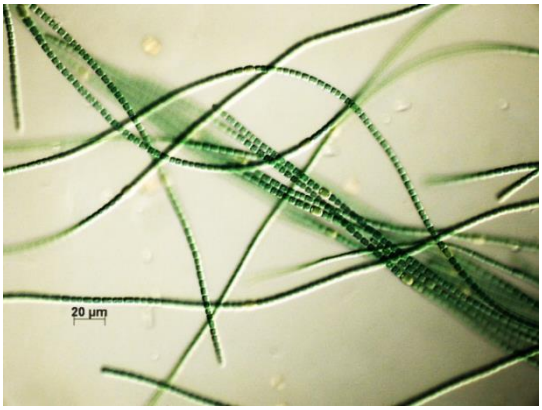
CAVN 17 (400x)

CAVN 20 (400x)

TVN40 (400x)



SRC (400x)



Bio 33 (400x)

Axioskop 2 *plus* (Carl Zeiss, Germany).

Figure II-1. Cultivated cyanobacterial strains.

II.1.2. Cultivation conditions

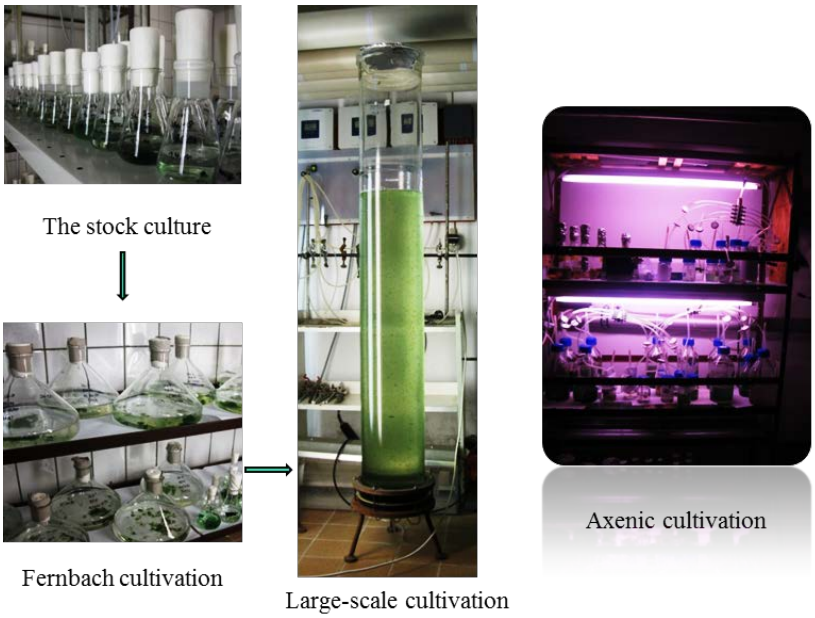


Figure II-2. Cultivation of cyanobacteria

II.1.2.1. *Media*

Chemicals and equipments:

- Sodium chloride (Carl Roth, Germany)
- Other chemicals (Merck/Carl Roth, Germany)
- Labor-Autoclave VARIOKLAV® (H + P Labortechnik GmbH, Germany)

The stock and micronutrient solutions were kept at 4°C until use. The mixed nutrition medium was autoclaved for 20 min at 121°C before use.

Table II-2. Composition of BG 11 medium

[Ripka and Herdman, 1993]

	Stock solution (g/100 mL)	Nutrient solution (mL)
$NaNO_3$	15.0	10.0
K_2HPO_4	0.31	10.0
$MgSO_4.7H_2O$	0.75	10.0
$CaCl_2.2H_2O$	0.36	10.0
Citric acid	0.06	10.0
Ferric ammonium citrate	0.06	10.0
EDTA (dinatrium-salt)	0.01	10.0

Na_2CO_3	0.20	10.0
Trace elements solution		1.0
Distilled water		919.0

Composition of the BG 11 trace elements solution [Kuhl and Lorenzen, 1964]:

	Stock solution (mg/100 mL)
H_3BO_3	6.1
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	28.7
$CuSO_4.5H_2O$	0.25
$(NH_4)_6Mo_7O_{24}.4H_2O$	1.25

BG 11 – N is BG 11 medium without $NaNO_3$. BG 11 + Co is BG 11 medium supplemented with Co^{2+} in the trace element solution. For cultivation of Bio 33, 0.5% NaCl was always added.

Composition of the BG 11 + Co trace elements solution:

	Stock solution (mg/100 mL)
H_3BO_3	6.1
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	28.7
$CuSO_4.5H_2O$	0.25
$(NH_4)_6Mo_7O_{24}.4H_2O$	1.25
$Co(NO_3)_2.6H_2O$	4.94

Table II-3. Composition of MBL medium

[Lorenzen and Guillard, 1972, modified according to Chu]

	Stock solution (g/50 mL)	Nutrient solution (mL)
$CaCl_2.6H_2O$	2.74	1
$MgSO_4.7H_2O$	1.85	1
$NaHCO_3$	0.63	1
K_2HPO_4	0.44	1
$NaNO_3$	4.25	1
$Na_2SiO_3.5H_2O$	1.06	1
Trace elements solution		1
Distilled water		993.0

TES	0.115 g/L medium

Composition of the trace elements solution:

	Stock solution (g/100 mL)
Na-EDTA	0.4360
$FeCl_3.6H_2O$	0.3150
$CuSO_4.5H_2O$	0.0010
$ZnSO_4.7H_2O$	0.0022
$CoCl_2.6H_2O$	0.0010
$MnCl_2.4H_2O$	0.0180
$Na_2MoO_4.2H_2O$	0.0006
H_3BO_3	0.1000

MBL – N is MBL medium without $NaNO_3$. MBL – Co is MBL medium without $CoCl_2 .6H_2O$ in the trace elements solution. For cultivation of Bio 33, 0.5% NaCl was always added.

II.1.2.2. *Equipments*

- 45 L glass column (JENAer Glas Rasotherm®, Germany)
- pH Measurement Ecoline® pH 170 (Wissenschaftlich – Technische Werkstätten GmbH, Germany)
- Heater Tetra*tec* HT 100 (230V/50Hz, Poland)
- 2 L Fernbach flasks (Merck, Germany)
- Erlenmeyer flasks 100 and 300 mL (Merck, Germany)
- Cellulose steristopper Nº. 18d, Nº. 29, 30 (Fisher Scientific GmbH, Germany)
- Centrifuge Rotanta 460R (Andreas Hettich GmbH & Co. KG, Germany)
- Centrifuge Stratos D37520 (Heraeus Instruments, Germany)
- Lyophylizer Alpha 1-4 (Christ GmbH, Germany)
- Filter paper 595 ½ Ø 185 mm (Schleicher & Schuell GmbH, Germany)
- Orbital shaker incubator OSFT-LS (TEQ GmbH, Germany)
- Incubation-shaking cabinet CERTOMAT® BS-1 (Sartorius, Germany),
- Orbital shaker Innova 2100 Platform Shaker (New Brunswick Scientific USA, USA)
- Cool-white fluorescent lamps (Radium NL 36W/21 – 840, Spectralux Plus Weiss, Germany)

II.1.2.3. *The stock culture*

For maintenance of laboratory culture, 2 – 3 mL of a 3-week-old cyanobacterial stock culture were inoculated in 50 mL BG 11 or MBL contained in 100 mL Erlenmeyer flasks. The cultivation was carried out at room temperature (18 - 20°C) and continuous illumination of 10 μmol photons m^{-2} s^{-1} by cool-

white fluorescent lamps. The stock cultures were shaken manually 2 times per week and renewed every 4 weeks.

II.1.2.4. *The Fernbach culture*

A volume of 5 mL of a 3-week-old cyanobacterial stock culture was inoculated into 50 mL medium contained in 100 mL Erlenmeyer flasks. After 2 to 3 weeks of incubation at room temperature (18 - 20°C), cyanobacteria were transferred to 300-mL flasks containing 100 mL medium. After 2 to 3 weeks, well-grown cyanobacteria were transferred into 2 L Fernbach flasks containing 1000 mL of medium. Fernbach flasks were incubated at room temperature under continuous fluorescent illumination and were manually shaken two times per week. Cool-white fluorescent lamps supplied a continuous irradiance of 10 μmol photons m^{-2} s^{-1}. After 2 to 3 weeks in culture, 1 Fernbach flask was partitioned into 10 Fernbach flasks.

Cyanobacteria were harvested after 35 days (Lincoln *et al.*, 1996) by Rotanta 460R centrifuge at 4100 rpm for 10 min at 20°C. The biomass was washed with distilled water, lyophilized and kept at -20°C until used. The culture medium was filtrated by filter paper and frozen at -20°C until used.

II.1.2.5. *Large-scale cultivation*

A volume of 10 mL of a 3-week-old cyanobacterial stock culture was inoculated in 100 mL medium contained in 200 mL Erlenmeyer flasks. After 2 to 3 weeks, these flasks were used as stock culture for the small-scale cultivation in 2 L Fernbach flasks containing 1000 mL medium. After 2 to 3 weeks, three Fernbach flasks were inoculated into a 45 L fermenter containing 15 L BG 11 medium. In the next 4 days, the fermenter was filled with 5 L medium every day until 35 L were achieved. The culture column was illuminated continuously with cool white fluorescent tubes at 20 μmol photons m^{-2} s^{-1}. Temperature was maintained at 22.5°C for strain Bio 33 and 26°C for strain TVN40 by using an aquarium heater. At 6^{th} day, the pH value of the large-scale culture was adjusted to 8.5 using CO_2 supplementation. During the cultivation, the culture was aerated with a mixture of CO_2 and air.

The cyanobacterial biomass was harvested after 35 days (Lincoln *et al.*, 1996) by Stratos D37520 centrifuge at 6500 rpm at 10°C. The biomass was washed with distilled water, lyophilized and kept at -20°C until used. The culture medium was stored in plastic cans at 4°C for immediately using (within 1 week) or frozen at -20°C until used.

II.2. Preparation of the extracts

II.2.1. Chemicals and equipments

- *n*-Hexane (GeReSo mbH, Einbeck, Germany)

- Ethyl acetate (GeReSo mbH, Einbeck, Germany)
- Methanol (GeReSo mbH, Einbeck, Germany)
- Sodium sulfate, water free (Merck KgaA, Germany)
- Laboratory sea sand p.a. (Merck KgaA, Germany)
- Porcelain mortar and pestle
- 250 mL and 500 mL Erlenmeyer flasks (Merck, Germany)
- System of rotatory vacuum evaporator: pump B-178, vacuum controller B-721, Rota vapor R-114, water bath B-480 (Büchi Labortechnik AG, CH)
- Centrifuge Rotana 96R (Hettich Zentrifugen GmbH & Co. KG, Germany)
- 100 mL centrifuge tube (Schott Duran, Germany)
- Freeze Lyophylizer Alpha 1-4 (Martin Christ Gefriertrocknungsanlagen GmbH, Germany)
- Filter paper 595 ½ Ø 185 mm (12 – 25 μm pore wide) (Schleicher & Schüll Microscience GmbH, Germany)
- Ultrasonic bath (Transsonic 460, Elma GmbH & Co KG, Germany)
- Magnetic stirrer (Variomag® Electronicrührer Mono H + P Labortechnik GmbH, Germany)
- Shaker (IKA-Labortechnik HS250 basic, IKA®-Werke GmbH & Co. KG, Germany)
- Thermo-plate DESAGA® 25-200°C (Sarstedt-Gruppe, Germany)

n-Hexane, ethyl acetate and methanol were distilled before use.

II.2.2. Extraction of cyanobacterial biomass and medium

II.2.2.1. *Extraction of biomass*

16 cyanobacterial strains shown in Figure II-1 were cultivated by the author in the years 2009 - 2010. The other biomasses named “old” biomasses (see Result, III.1), were received from the collection of Department of Pharmaceutical Biology, Institute of Pharmacy, Ernst-Moritz-Arndt University Greifswald, Germany. Those cyanobacterial strains were cultivated before 2008.

1 g of dried biomass was crushed with sea sand and a small portion of the 150 mL *n*-hexane in a mortar with pestle to get a fine suspension. The rest of *n*-hexane was added and the cells were broken again in ultrasonic bath for 7 min. Then, the biomass was extracted under magnetic stirring for 2 h at room temperature. The supernatant was separated from the residue by centrifugation at 4100 rpm for 10 min at 10°C. This step was repeated three times. The rest of *n*-hexane was removed by incubation on a thermo-plate at 40°C. The biomass residue was dried overnight and further extracted with 3 x 150 mL MeOH followed by 150 mL water. Rotary evaporator followed by lyophilization was used to

remove the solvents completely. The MeOH extract and H_2O extract were weighed and kept at -20°C until use.

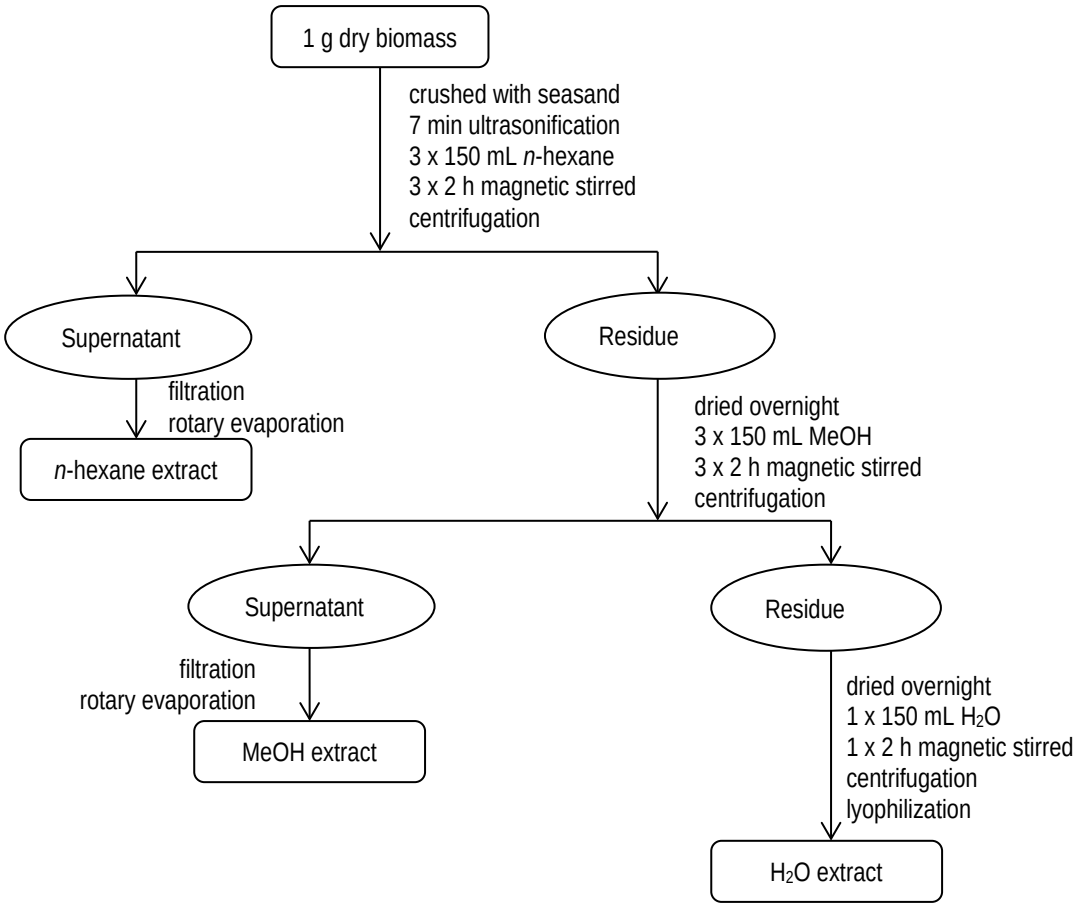


Figure II-3. Extraction of cyanobacterial biomass for screening experiments.

II.2.2.2. *Extraction of medium with EtOAc*

A volume of 3 L culture medium was reduced to 300 mL by rotary evaporator (40°C). 300 mL of EtOAc were added and the mixture was shaken for 24 h. The EtOAc layer was separated from the aqueous layer. The aqueous layer was shaken again with 300 mL of fresh EtOAc. The EtOAc layers were combined, dried over Na_2SO_4 and rotary evaporator was used (40°C) to remove the organic solvent. The rest of EtOAc was removed on the thermo-plate at 40°C. The aqueous layer was discarded. The extracts were weighed and kept at -20°C until use.

II.3. Screening for antimicrobial and cytotoxic activity

II.3.1. Culture media

Table II-4. Composition of standard II nutrient agar

(Merck, Germany)

	Amount (g/L)
Peptone from meat	3.45
Peptone from casein	3.45
NaCl	5.1
Agar-agar	13.0

25 g mixture of standard II nutrient agar was suspended in 1 L distilled water, mixed thoroughly and autoclaved at 121°C for 20 min.

Table II-5. Composition of Malt agar

	Amount (g/L)
Malt extract (Glucose 10-15%, Maltose 40-50%, Dextrin 10-15%, Protein 5%) (Dr. Fränkle and Max Eck, Fellbach, Germany)	30.0
Agar-agar (Carl Roth, Germany)	15.0

Malt extract and Agar-agar were weighed and the mixture was suspended in 1 L distilled water, mixed thoroughly and autoclaved at 121°C for 20 min.

Table II-6. Composition of Mueller Hinton II agar

(Becton, Dickinson & Company, France)

	Amount (g/L)
Beef extract	2.0
Acid hydrolysate of casein	17.5
Starch	1.5
Agar	17.0

38 g mixture of Mueller Hinton II agar were suspended in 1 L distilled water, mixed thoroughly and autoclaved at 121°C for 20 min.

II.3.2. Test-organisms

The *n*-hexane, methanol, water and ethyl acetate extracts were tested for antibiotic activity against Gram-positive bacteria *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6051, Gram-negative bacteria *Escherichia coli* ATCC 11229, *Pseudomonas aeruginosa* ATCC 27853 and the

yeast *Candida maltosa* SBUG 700 by agar diffusion assay. The extracts were used in a dose of 2 mg/6 mm paper disc. The experiment was repeated 3 times (2 parallels). Inhibition zone was measured including the diameter of the paper disc.

The bacteria were maintained on Mueller Hinton II agar plate and the yeast was maintained on Malt agar. They were renewed every four weeks. Before antimicrobial or antifungal test, the organisms were streaked out on fresh agar plates and incubated at 37°C (for bacteria) or 26°C (for yeast) over 18 – 24 h.

In addition, the main fraction FIII (see II.4.2) (1 mg/6 mm paper disc) was tested against the human pathogenous yeasts and fungi *Candida albicans* ATCC 90028, *Candida krusei* ATCC 90878, *Aspergillus fumigatus, Microsporum gypseum*, *Microsporum canis*, and *Mucor* sp.. These tests were carried out by Gudrun Schröder (Friedrich Loeffler Institute of Medical Microbiology, University Medicine Greifswald).

II.3.3. Agar diffusion test

II.3.3.1. *Materials*

- Nystatin (Carl Roth, Germany)
- Gentamycin sulfate (Biochrom AG, Germany)
- Ampicillin (Merck KGaA, Germany)
- Sterile plastic Petri dishes (Ø 90 mm) (VWR, Germany)
- Incubator Mytron BS 120 (Memmert, Germany)
- Metal pins (1 x 20 mm)
- Sterile antibiotic test paper discs Ø 6 mm (Schleicher & Schüll Microscience GmbH, Germany)
- NaCl 0.9% (Merck KGaA, Germany)
- Laminar flow box (Heraeus Instruments, Germany)
- Plastic inoculating loop, sterile (VWR, Germany)
- 10 mL disposable serological pipettes (VWR, Germany)
- Sterile tips 1-200 μL, 100 – 1000 μL (Carl Roth, Germany)
- Polystyrene plate (20 x 20 cm)
- Beaker glass (Merck, Germany)
- Iodonitrotetrazolium chloride (INT) (Sigma-Aldrich Chemie GmbH, Germany): 5 mg of the reagent was dissolved in 1 mL of ethanol 50% to get a spraying solution.
- Bacteria: see II.3.2.

II.3.3.2. *Method*

Table II-7. Concentration of the positive control in agar diffusion test

Substance	Amount (μg/paper disc)	Tested organism
Nystatin	10	*Candida maltosa*
Ampicillin	10	*Bacillus subtilis*
Ampicillin	10	*Staphylococcus aureus*
Ampicillin	50	*Escherichia coli*
Gentamicin	25	*Pseudomonas aeruginosa*

Method was done according to Mundt *et al.* (2001). 2 mg of the extract or 1 mg of the fraction of the isolation procedure (see II.4.2) were dissolved in 50 μL of the extracting solvent. The solutions were dropped on paper discs. The discs were fixed by pins on a polystyrene plate for drying 1-2 h under sterile condition. A volume of 20 mL nutrient agar was cooled down to 40°C in a sterile beaker glass. One full loop of a stock culture of bacteria was suspended in 3 mL NaCl 0.9% and mixed thoroughly. 200 μL of this bacterial suspension were mixed well in chilled nutrient agar (about 40°C). The mixture was poured into a Petri dish. After solidification of the agar, the solvent-free paper discs containing cyanobacterial extracts or fractions were placed on the surface of the agar. The petri dish was kept at 4°C about 3 h for prediffusion. Afterwards, the plates were incubated for 24 h at 37°C for bacteria and at 26°C for *Candida maltosa* in an inverted position. Negative control experiments were performed by using paper discs loaded only with an equivalent volume of the solvent and positive control experiments were performed with antibiotics shown in Table II-7. At the end of the incubation period, the inhibition zones were measured and expressed as the diameter of the clear zone including the diameter of the paper disc (∅ 6 mm).

II.3.4. Minimum inhibitory concentration (MIC)

II.3.4.1. *Materials*

- Incubator Mytron BS 120 (Memmert, Germany)
- 20 – 50 mL beaker glass (Merck, Germany)
- 50 mL Erlenmeyer flasks (Merck, Germany)
- Sterile Petri dishes (Ø 90 mm) (Carl Roth, Germany)
- 96 well microtiter plate (TPP, Switzerland)
- 4 canal and 8 canal pipettes and tips (Biohit Proline, Finland)
- Shaker waterbath GFL 1083 (Gesellschaft für Labotechnik, Germany)
- FLUOstar Omega microplate reader (BMG LABTECH GmbH, Germany)

- Sterile NaCl 0.9% (Merck KGaA, Germany)
- Iodonitrotetrazolium chloride (INT) (Sigma-Aldrich Chemie GmbH, Germany): see II.3.3.1.

Table II-8. Composition of PBS buffer

(PBS Dulbecco, Biochrom AG, Germany)

	Amount (g/100 mL)
NaCl	8.0
KCl	0.2
KH_2PO_4	0.2
Na_2HPO_4	1.15

9.55 g dry PBS was dissolved in 100 mL distilled water. The solution was autoclaved at 121°C for 20 min.

Table II-9. Composition of Bouillon broth

(Merck, Germany)

	Amount (g/L)
Peptone from meat	5.0
Meat extract	3.0

8 g dry powder was dissolved in 1 L distilled water. The solution was autoclaved at 121°C for 20 min.

II.3.4.2. *Method*

The minimum inhibitory concentration of the main fraction FIII (see II.4.2) was determined by broth dilution method using 96-well microtiter plates against *Candida maltosa* SBUG 700. MIC value of the positive control nystatin against *Candida maltosa* SBUG 700 was estimated as 1.02 μg/mL. The yeast *Candida maltosa* was renewed 22 h before use. For the broth dilution method, the suspension A of the yeast was prepared by suspending a loop of the renewed *Candida maltosa* in 2 mL of sterile NaCl 0.9%. 100 μL of suspension A was inoculated into 10 mL of sterile Bouillon broth (the suspension B). B was shaken at 26°C, 125 rpm for 22 h in a water bath. After incubation, B was diluted 1/100 with sterile NaCl 0.9%. Then, 1 mL of the suspension was mixed well with 10 mL Bouillon solution to create the final suspension for the MIC estimation (the suspension C).

The main fraction FIII of Bio 33 (see II.4.2) was dissolved in PBS to get stock solutions C1 (2.75 mg/mL) and C2 (2.5 mg/mL). Each stock solution was tested in four parallels. 200 μL of each stock solution were pipetted into the 1st row of the 96-well plate (C1 from A1 to D1 and C2 from E1 to H1).

100 µL of PBS was pipetted into all other wells from 2nd to 12th row of the micro titer plate except the well H12. The two-fold serial dilution was done by pipetting 100 µL of the first row into the corresponding well of the second row and so on until the 11th row was reached. The 100 µL of the 11th row were discarded. The final concentrations were between 1375 (or 1125) and 1.343 µg/mL (or 1.099 µg/mL).

100 µL of the suspension C was then pipetted into all wells from 1st row to 11th row. The wells A12 to D12 were filled with 100 µL PBS and 100 µL suspension C as negative controls (Y-contr.). 100 µL PBS and 100 µL Bouillon broth were added into the wells E12 to G12 as medium controls (B-contr.). The well H12 was kept empty as photometric blank. The diagram of the 96-well plate is shown in Figure II-4.

	C 1	C/2 2	C/2^2 3	C/2^3 4	C/2^4 5	C/2^5 6	C/2^6 7	C/2^7 8	C/2^8 9	C/2^9 10	C/2^{10} 11	Control 12
A	C1/2											Y-contr.
B	C1/2											Y-contr.
C	C1/2											Y-contr.
D	C1/2											Y-contr.
E	C2/2											B-contr.
F	C2/2											B-contr.
G	C2/2											B-contr.
H	C2/2											Blank

Y-contr.: control included yeast + Bouillon broth + PBS; B-contr.: control included bouillon broth +PBS. Concentration C1/2 and C2/2 were the highest concentrations of the FIII main fraction in the wells A1-D1 (C1) and wells E1-H1 (C2) of finished test plate.

Figure II-4. Preparation of the microtiter plate for MIC estimation against *Candida maltosa*.

The plate was incubated at 26°C for 22 h. After the incubation, 20 µL of INT (*p*-iodonitrotetrazolium violet, 2.5 mg/ml 50% EtOH) was added to each well and the plate was incubated for 30 min. The OD was measured using FLUOstar Omega microplate reader at 550 nm.

The MIC was estimated as the lowest concentration of the tested compound that still inhibited the yeast growth. The inhibition curve was plotted based on the OD values of wells containing yeast cultivated in the presence of different concentrations of tested substances in relation to wells containing yeast cultivated without tested substances. Based on the inhibition curve, the minimal inhibition concentration of a given substance was determined as the average of the last concentration in which yeast cells can growth and the first concentration in which they did not grow.

II.3.5. Cytotoxicity assay

Cytotoxicity assay of extracts and fractions of strain Bio 33 was performed by the Cell working group, Pharmaceutical Biology, Institute of Pharmacy, Ernst-Moritz-Arndt University Greifswald, Germany.

II.3.5.1. *Chemicals and equipment*

- Solution:
  A. Swelling buffer:
     20 mM Tris-buffer substance (242 mg) (PUFFERAN®, Carl Roth, Germany)
     1 mM $MgCl_2.6H_2O$ (20 mg) (Carl Roth, Germany)
     0.5 mM $CaCl_2.6H_2O$ (11 mg) or $CaCl_2.2H_2O$ (7 mg) (Carl Roth, Germany)
     Add 100 mL of deionized water (PAA, Austria) and adjust pH to 7.4
  B. Lysis buffer:
     5% Benzalkonium chloride (0.5 g) (Sigma-Aldrich Chemie GmbH, Germany) in 0.3 mL 3% acetic acid (Carl Roth, Germany)
     Add cell culture water (PAA, Austria) to 10 mL
  C. Trypsin/EDTA solution (Biochrom AG, Germany) (10x), 0.5%/0.2% (w/v), w/o Ca^{2+}, w/o Mg^{2+}, dilute 1:10 with cell culture water (PAA, Austria)
  D. PBS/EDTA solution:
     Fill up 5 mL 0.1 M EDTA solution (preparation: dissolve 3.772 g EDTA-$Na_2.2H_2O$ (Sigma-Aldrich Chemie GmbH, Germany) in 100 mL cell culture water (PAA, Austria)) with Dulbecco's PBS (PAA, Austria) (1x, w/o Ca^{2+}, w/o Mg^{2+}) to 100 mL. Filtersterilized with 0.22 μm Whatman filter (Whatman, Germany)
  E. RPMI medium with 10% FCS:
     Roswell Park Memorial Institute Medium 1640 (RPMI 1640) with L-Glutamine (BioWhittaker®, Lonza, Belgium)
     Add 1% Penicillin/Streptomycin solution (10000 U/10000 μg/mL) (Sigma-Aldrich Chemie GmbH, Germany)
     Add 10% v/v FCS (fetal calf serum) (Sigma-Aldrich Chemie GmbH, Germany)
  F. Neutral red (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride) (Sigma-Aldrich Chemie GmbH, Germany)
     Stock solution: 0.33 g neutral red in 100 mL cell culture water (PAA, Austria)
     Used solution for 1 plate: 120 μL stock solution/12 mL medium RPMI (with 10% FCS) (see above)
  G. Ethanol/Acetic acid: 1% acetic acid, 50% EtOH and 49% deionized water.
  H. Dilution of test substance (extracts)
     250 μg/mL (end concentration in the well), dilute 1:2 with medium (8 dilutions); possible: stock solution of 20 mg/mL in EtOH (EtOH/H_2O, DMSO) dilute with medium
  I. Positive control: Etoposide (Sigma-Aldrich Chemie GmbH, Germany)

Stock solution: 80 μM

Used solution: 0.5 μM per well

- HBSS (Hanks' Balanced Salts Solution) (PAA, Austria) 1x, with Ca^{2+}, Mg^{2+}, w/o phenol red
- Acetic acid 100% (Carl Roth, Germany)
- Ethanol 96% (Merck KgaA, Germany)
- Cell culture water (AP), EP Grade, steril (PAA, Austria)
- 5637 cell line: human urinary bladder cancer cell line (German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany))
- 96 well micro titer plate – cell culture (Biochrom AG, Germany)
- 8 channel pipette and tips (Biohit Proline, Finland)
- 50 mL plastic flasks for cell culture (Biochrom AG, Germany)
- Incubator Mytron BS 120 (Bio- und Solartechnik GmbH, Germany)
- 5 mL glass Pasteur pipette (Merck, Germany)
- Manger (or Petri dish) for 4 – 8 channel pipettes (Merck, Germany)
- Shaker IKA-Vibrax-VXR (IKA Labortechnik, Germany)
- Bürker cell counting chamber 0.1 mm; 1/25 + 1/400 mm^2 (Bürker fein-Optik Bad, Germany)
- FLUOstar Omega microtiter readers (BMG LABTECH GmbH, Germany)

II.3.5.2. *Method*

Cytotoxic activity was determined by Neutral Red Uptake Assay (NRU-Test) according to Bohrenfreund and Puerner (1985) with the cultivated human bladder carcinoma cell line 5637 (ATCC HTB-9) used for the assay.

For cytotoxicity assay, the 5637 cells were seeded in a 96-well plate one day before testing as described in the following. The old medium RPMI (with 10% FCS) from a T25 cm^2 cell culture flask seeded with the 5637 cell line was decanted. The cells were rinsed by shaking the flask carefully for 30 s with 1 mL PBS/EDTA. The solution was decanted. 1 mL of PBS/EDTA was added and the flask was incubated for 10 min at 37°C. After that, the solution was decanted. 1 mL of Trypsin/EDTA was added and the flask was incubated for 3 min at 37°C.

After incubation, 3 mL of RPMI medium (with 10% FCS) were added. The cells were detached from the bottom of the flask by rinsing and transferred into a centrifuge glass. The cells were centrifuged at 1000 rpm/min for 3 min and the supernatant was carefully decanted. 2 mL of RPMI medium (with 10% FCS) were added and mixed well. 20 μL of prepared cell suspension was mixed with 460 μL of swelling buffer in an Eppendorf tube, incubated for 7 min at room temperature. Then, 20 μL of lyses

buffer were added and mixed well (shaker). After the cell suspension was prepared, subculture was prepared in new T25 or T75 flask.

For seeding of one plate, 8 mL of RPMI medium (with 10% FCS) containing 0.24 Mio cells (3000 cells/well) was required. 100 µL of cell suspensions was added into the 2nd to 11th column; 2nd to 7th row (the wells from 2B to 11G as shown in Figure II-5), mixed well and then incubated for 24 h.

For cytotoxic assay: After 24 h of incubation, the medium was removed and 100 µL of new RPMI medium (with 10% FCS) was added to the 3rd and 11th column; 150 µL into all other wells. Etoposide as positive control was added in a volume of 50 µL in the wells of the 3rd column; 50 µL of solvent as control in the 11th column and 150 µL of test substance solution with the highest concentration in the 4th column.

	1	2	3	4	5	6	7	8	9	10	11	12
A	RPMI	Cell	Etop-osid	Extract C1	C1/2	C1/2^3	C1/2^4	C1/2^5	C1/2^6	C1/2^7	Solvent	RPMI
B			0.5 µM									
C												
D												
E												
F												
G												
H												

Figure II-5. The 96 well plate for cytotoxicity assay.

C1: Highest tested concentration of substance.

without cells
with cells

The test substance in the 4th column was mixed well and then diluted 1:2 fold to afford the substance concentration of 1:2 (C2), 1:4 (C3), etc. by pipetting 150 µL of C1 to C2, C2 to C3 and so forth (C1-C7 as shown in Figure II-5). The cell culture microtiter plate was further incubated for 72 h at 37°C.

After incubation, the cells were washed with 200 µL of HBSS. New RPMI medium (with 10% FCS) containing NRU solution was added. Cells were incubated for 3 h at 37°C. The supernatant of the cell culture was then removed carefully. The cells were rinsed twice using 100 µL of HBSS. 100 µL of EtOH/acetic acid were added into the wells and the microtiter plate was subsequently agitated for 30 min at room temperature. The OD of the cell culture was measured using a FLUOstar Omega microtiter reader at wavelength of 540 nm.

The cytotoxicity was determined based on the OD of the test wells in relation to the solvent control and the cell control after staining with neutral red. The percentage of living cells was calculated by comparison of the OD of wells containing the test solutions with the solvent controls and positive control according to the equation see below:

$$\text{Living cells [\%]} = [(OD_{test} - OD_{blank})*100]/ (OD_{control} - OD_{blank})$$

The cytotoxicity of substances was determined as percentage of reduced cell number in presence of the test substance as following:

$$\text{Cytotoxicity [\%]} = 100 - \text{living cells [\%]}$$

II.4. Bioassay-guided fractionation of the crude extracts

The isolation of the active compounds from the biomass of strain Bio 33 is summarized in Figure II-6.

II.4.1. Preparation of the crude extract

Initially, 2.5 g dried biomass was crushed with sea sand and a small portion of *n*-hexane in a mortar with pestle to get a fine suspension. 250 mL *n*-hexane were added to the suspension followed by homogenization for 7 min in ultrasonic bath. After this, the biomass was extracted under stirring for 2 h at room temperature. The supernatant was separated from the residue by Rotanta 460R centrifuge at 4100 rpm for 10 min at 4°C. The residue was extracted with *n*-hexane three times and dried overnight. The dried residue was further extracted with MeOH/H_2O (1:1, v/v) three times applying the above method. The organic solvents were reduced with the rotary evaporator and the rest of water was removed completely by lyophilization. The MeOH/H_2O extract was weighed and kept at -20°C until use.

II.4.2. Fractionation of MeOH/H_2O extract by silica gel column chromatography

II.4.2.1. *Materials*

- Sample: 600 mg MeOH/H_2O crude extract/column
- Glass column with a frit: 26 x 2.2 cm
- 1 L round bottom flasks (Merck, Germany)
- Stationary phase: 55 g silica gel for column chromatography (0.040 - 0.063 mm) (Merck, Germany)
- Fraction Collector Model 2110 (Bio-Rad Laboratories, USA)
- 5 mL glass tubes
- Mobile phase EtOAc/MeOH/H_2O in different ratio 7:2:1, 5:3.5:1.5, 1:7:2 and 5:5

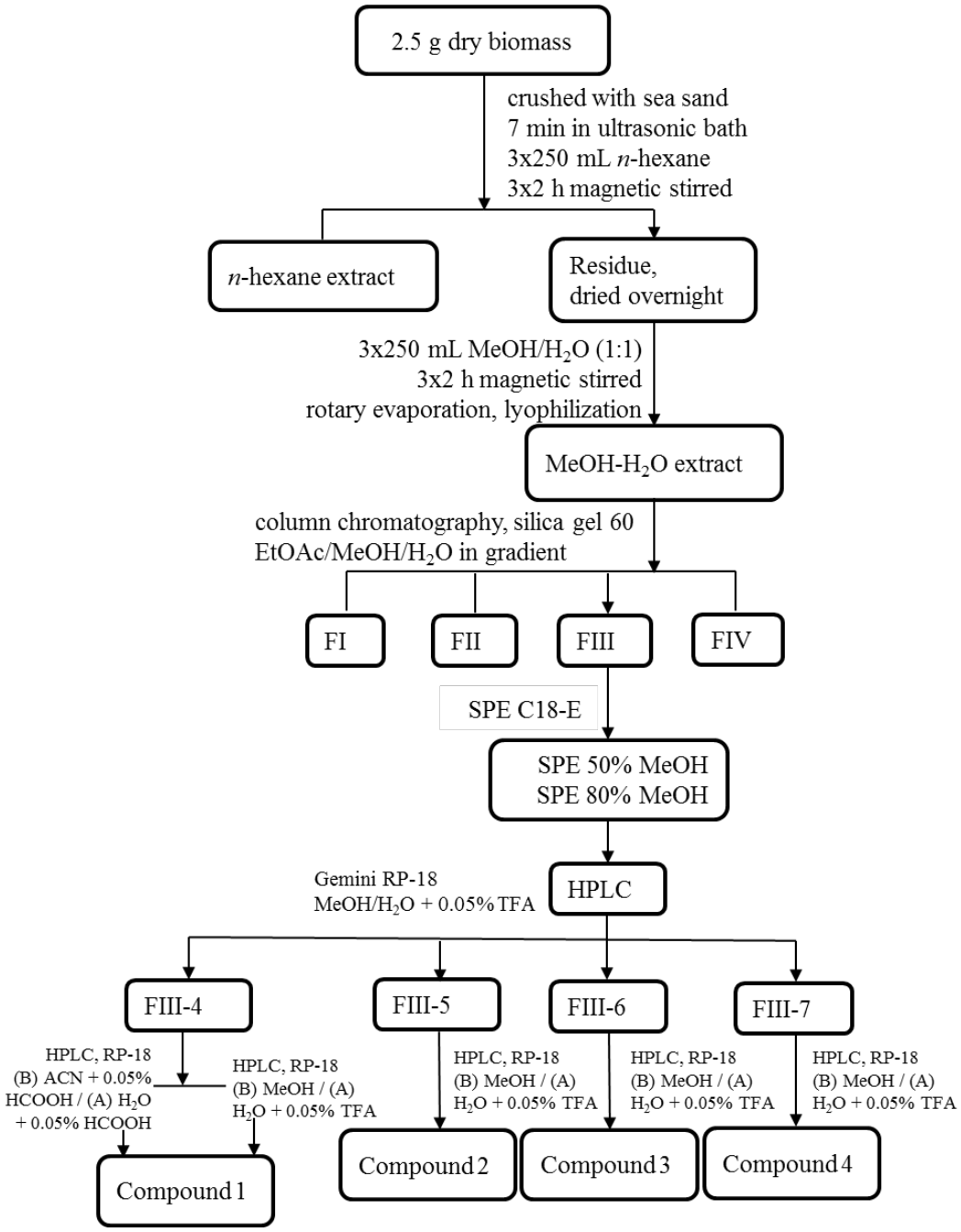


Figure II-6. Diagram of isolation steps of balticidins from the biomass of strain Bio 33.

II.4.2.2. *Method*

Silica gel was mixed and saturated in the first solvent system SS1 (Table II-10). The suspension was then carefully poured into the column to prevent any air bubble in the column. A 0.5 cm thick layer of sea sand was filled on top of the silica gel column. Initially, the silica gel column was stabilized with the first organic solvent for 30 min. The column flow was then adjusted to 3 mL/5 min. 600 mg of the sample were mixed and crushed with 600 mg sea sand and applied dry on the surface of silica gel bed. The mobile phase was continuously added in the order of solvent system SS1, SS2, SS3, SS4 (Table II-10).

For elution of unpolar compounds, 200 mL of SS1 was used and FI was collected as a whole. The outflow of FII fraction eluted with SS2 was collected in volume of 3 mL automatically by the fraction collector. The received 3 mL fractions were analyzed by TLC using the SS2 eluting solvent system, detected with UV light at 254 nm, 356 nm and by spraying with anisaldehyde sulphuric acid reagent. Fractions with the same spots on TLC were combined to 3 main fractions FII-1, FII-2, FII-3. The outflows of solvent system SS3 (FIII) and SS4 (FIV) were taken as a whole in round bottom flasks.

With the aim of identifying antifungal active fractions, all received fractions FI (SS1), FII-1, FII-2, FII-3, FIII (SS3) and FIV (SS4) were tested against *Candida maltosa*. From the results of the agar diffusion assay, the active fractions FII-3 and FIII were combined for further isolation of the active compounds. The other fractions exhibited no activity against *Candida maltosa*.

Table II-10. Solvent systems used in column chromatography for separation of MeOH/H_2O extracts

Fraction	Volume (mL)	Solvent system	Solvent	Ration
FI	200	SS1	EtOAc/MeOH/H_2O	7:2:1
FII	150	SS2	EtOAc/MeOH/H_2O	5:3.5:1.5
FIII	500	SS3	EtOAc/MeOH/H_2O	1:7:2
FIV	400	SS4	EtOAc/MeOH/H_2O	5:5

II.4.3. Purification of the main fraction by solid phase extraction (SPE)

Material:

- STRATA® C18-E Giga™ tubes (55 μm, 70 Å, 5 g/20 mL, Phenomenex, Germany)
- SPE 12 Port vacuum manifold (SPE-12G, J. T. Baker, USA)
- Pump (KNF lab, LABOPORT, USA)

The sorbent bed of the SPE cartridge was activated with 50 mL of MeOH. Afterwards, 20 mL of H_2O was added to equilibrate the sorbent. To remove the undesired polar compounds, 100 mg of fraction FIII was suspended in 5 mL of 20% MeOH/H_2O and loaded on the SPE cartridge. The cartridge was successively eluted with 20%, 45%, and 80% MeOH in H_2O followed by 100% MeOH, each 100 mL. The fractions were collected separately, rotary evaporated to remove the organic solvent and lyophilized.

The SPE cartridge was washed with 100 mL MeOH and 100 mL acetone to remove the unpolar compounds. After being dried, the cartridge was reactivated with MeOH.

II.4.4. Thin layer chromatography (TLC)

II.4.4.1. *Materials*

- Aluminum TLC plates Silica gel 60 F_{254}, 20x20 cm, 0.2 mm (Merck, Germany)
- Anisaldehyde- sulphuric acid (AS) reagent: 10 mL 10% H_2SO_4 (Carl Roth Germany) is added to 50 mL acetic acid (Carl Roth, Germany) containing 0.5 mL anisaldehyde (Carl Roth, Germany)
- Thermo-plate DESAGA® 25 - 200°C (Sarstedt-Gruppe, Germany)
- Desaga TLC-Lightbox HP-UVIS 254/366 nm (DESAGA, Germany)
- Kamera Canon PowerShot S 2 IS (Canon U.S.A. Inc., USA)
- Glass chamber for TLC (Camag, Switzerland)
- Organic solvents:
  - methanol (GeReSo mbH, Einbeck, Germany)
  - dichloromethane (GeReSo mbH, Einbeck, Germany)
  - ethyl acetate (GeReSo mbH, Einbeck, Germany)
  - water (Reinstwasseranlage SG-ClearUV, SG Wasseraufbereitungs- und Regenerierstation GmbH, Germany)

II.4.4.2. *Method*

For analytical purpose, the thin layer chromatography sheet (20x20 cm) was cut into smaller sheets (6.5x6.5 cm or 6.5x2 cm). The start line was marked 1cm above the bottom edge of the sheet and running distance was 5 cm. The sample was applied on the start line of TLC sheet as a thin line. The sheet was developed in a 9x3x9 cm glass chamber containing 10 mL solvent mixture. The migration of a substance was determined by the R_f value.

The following solvent systems were used for analytical purpose.

Mobile phase:

- ethyl acetate: methanol: water (7:2:1)
- ethyl acetate: methanol: water (5:3.5:1.5)
- ethyl acetate: methanol: water (1:7:2)
- methanol: water (8:2)

The chromatograms were detected at 254 nm and 365 nm UV light and by spraying with anisaldehyde sulphuric acid reagent and heated at 110°C for 5 min.

II.4.5. Bio-autochromatography

II.4.5.1. *Materials*

- Aluminum TLC plate Silica gel 60 F_{254}, 20x20 cm, 0.2 mm (Merck, Germany)
- Petri dishes 20 – 50 mL (Ø 90 mm) (Merck, Germany)
- Incubator Mytron BS 120 (Memmert, Germany)
- 50 mL sterile beakers (Merck, Germany)
- Laminar flow box (Heraeus Instruments, Germany)
- Sterile NaCl 0.9% (Carl Roth, Germany)
- Nutrient agar II (Merck, Germany) (Table II-4)
- *Candida maltosa* SBUG 700
- Iodonitrotetrazolium chlorid (INT) (Sigma-Aldrich Chemie GmbH, Germany) 2.5 mg/ mL ethanol 50%
- Organic solvents: see TLC method (II.4.4.1)
- Mobile phases: see TLC method (II.4.4.1)

II.4.5.2. *Method*

The TLC bioautographic method was used for detection of fractions containing antifungal compounds in the crude extract or the main fraction. This assay based on the agar diffusion technique.
2 mg of the MeOH, MeOH/H_2O crude extracts or 500 μg of the main fraction FIII were applied onto the analytical TLC plate and developed with different solvent systems. The developed TLC sheets after being dried carefully were fixed in the Petri dish containing 10 mL solid nutrient agar II. 20 mL nutrient agar II solution inoculated with 200 μL *Candida maltosa* suspension (prepared by dissolving one full loop of *Candida maltosa* stock culture in 2 mL NaCl 0.9%) was poured onto the surface of developed TLC sheets carefully to prevent the development of air bubbles on the surface of TLC sheets. The Petri dish was incubated at 4°C for 2.5 h and at 26°C over night (16 – 18 h). Clear zones on the agar surface indicate antifungal activity. These zones became more visible against a dark pink background by spraying the agar surface with INT reagent. The control chromatogram was detected at 254 nm and 365 nm UV light and by spraying with anisaldehyde sulphuric acid reagent.

II.4.6. Purification of the active compounds by HPLC

II.4.6.1. *Equipments*

- HPLC column: reversed phase Gemini C18 column (Phenomenex, USA): 110 Å, 250x4.6 mm, 5 μm; LiChrospher RP-18e (Merck, Germany): 100 Å, 250x4.0 mm, 5 μm
- Methanol HPLC (gradient grade, Merck, Germany)
- Deionized water (Pure water system Clear UV plus, SG Water Preparation and Recycling GmbH, Germany)
- TFA (for spectroscopy, Merck, Germany)
- HCOOH (for spectroscopy, Merck, Germany)
- HPLC system:
  - Kontron Instruments (KONTRON, Italy)
    - Diode array detector (DAD 440)
    - Auto sampler (SA360)
    - Pump 422 & 422S
    - Geminix software, version 1.91
  - SHIMADZU LC-20a prominence UFLC (SHIMADZU Corporation, Japan)
    - Diode array detector SPD-M20A
    - Auto sampler SIL-10AF
    - Pumps LC-20AD
    - Control unit CBM-20A
    - Column oven CTO-10AS
    - LCsolution software

II.4.6.2. *Analytical HPLC*

The main fraction FIII and others active fractions were analyzed by analytical reversed phase HPLC using Gemini C18 column and the mobile phase MeOH in H_2O plus 0.05% TFA, flow rate 1 mL min^{-1} (program 1 and program 2). The separation was controlled at 210, 226, 238, 254 nm by DAD. A sample concentration of 4 mg/mL was used. A sample volume of 20 μL was injected.

HPLC program 1:

Time (min)	0.5	30.5	40.5	42.5	46.0
% MeOH	10	100	100	10	10

HPLC program 2:

Time (min)	0.0	0.5	4.5	7.5	30.5	35.5	37.5	42.5
% MeOH	40	40	60	70	100	100	40	40

II.4.6.3. *Fractionation of FIII*

From SPE fractions (45% and 80% MeOH fractions) of FIII four peaks were collected using a LiChrospher RP-18e column and the mobile phase MeOH/H_2O + 0.05% TFA (program 3) with a flow rate of 1 mL min^{-1}. A concentration of 200 μg/20 μL was injected per run. The separation was detected at 226 nm by DAD.

HPLC program 3:

Time (min)	0.5	4.5	7.5	26.0	28.0	33.0	35.0	37.5
% MeOH	40	60	74	74	100	100	40	40

II.4.6.4. *Purification of isolated fractions*

To purify the isolated fraction FIII-4, the mobile phase MeOH/H_2O plus 0.05% TFA with a flow rate of 1 mL min^{-1} (program 4) was used. The column LiChrospher RP-18e was used as stationary phase. The separation was controlled at 210, 226, 238, 254 nm by DAD. The sample concentration was 160 μg/40 μL per run.

HPLC program 4:

Time (min)	0.5	2.5	17.5	19.0	23.5
% MeOH	60	70	90.4	60	60

In parallel, the pure compound 1 was isolated from FIII-4 with the mobile phase ACN plus 0.05% HCOOH in H_2O plus 0.05% HCOOH (HPLC program 5). The same column, flow rate and sample concentration as above were used. The separation was controlled at 226 nm by DAD.

HPLC program 5:

Time (min)	0.50	5.0	7.5	17.5	20.0	23.0	24.0
% ACN	30	35	37	37	40	40	30

Fractions FIII-6 and FIII-7 were further purified using an isocratic mobile phase with 74% MeOH in H_2O plus 0.05% TFA (HPLC program 6), flow rate 1 mL min^{-1} with a LiChrospher RP-18e column. A concentration of 50 μg/20 μL was injected per run. The separation was detected at 226 nm by DAD.

II.5. Structural elucidation of the isolated compounds

UV spectra were recorded on a LC-20a prominence UFLC (SHIMADZU Corporation, Japan) with a diode array detector SPD-M20A. The IR spectra were recorded using a NICOLET IR200 Fourier transform infrared spectrometer (FT-IR) (Thermo Electron Comporation, USA).

Structural elucidation was executed at the Helmholtz Centre for Infection Research, Braunschweig (Germany).

1D and 2D NMR spectra were recorded at 300 K on a Bruker AVANCE DMX 600 NMR spectrometer (COSY, TOCSY, NOESY, HMQC and HMBC) and a Bruker AVANCE 600 NMR spectrometer (COSY, TOCSY, NOESY, HSQC, 2D-edited HSQC, HSQC-TOCSY and gHMBC) equipped with an UltraShield Plus magnet and a triple resonance cryoprobe with gradient unit. Each spectrometer was locked to the deuterium resonance of the solvent, DMSO-d_6 or trifluoroethanol-d_2/H_2O (1:1). Chemical shifts were referenced to the residual signals of the respective solvents (DMSO ^{1}H: 2.50 / ^{13}C: 39.5 ppm and CF_3*C*D*H*OH ^{1}H: 3.95 / ^{13}C: 61.5 ppm, respectively).

ESI MS^n. The compounds were analyzed on an Orbitrap Velos mass spectrometer (Thermo-Scientific, Bremen, Germany) equipped with a nanospray ion source and an external high energy collision cell. MS/MS spectra of the doubly protonated molecular ions were recorded at a resolution of 60 k and with an average mass error of under 2 ppm. The most probable isotopic compositions of all major fragment ions were determined, followed by their manual structural assignment. If necessary, MS^3 and higher order experiments were performed for corroboration of structural assignments.

Carbohydrate compositional analysis. Monosaccharides were analyzed as the corresponding methyl glycosides after methanolysis and trimethylsilylation by GC/MS (Chaplin, 1982). The absolute configuration of the monosaccharides was determined by separation of the trimethylsilylated S-(1)-but-2-yl glycosides (Gerwig *et al.*, 1978).

Enantioselective analysis of amino acids. A sample of compound 1 and 2 respectively was hydrolyzed using 4 N TFA under various temperature-time regimes (80°C: 2 and 5h; 100°C: 3h), conditions that resulted in the conversion of Gln to Glu. After drying, the resulting free amino acids were derivatized with 4 N HCl/propan-2-ol (1 h, 110°C) and, after removal of reagents, the amino acid isopropyl esters were then acylated with pentafluoropropionic acid anhydride in dichloromethane (150°C, 12 min). Excess reagents were again removed and the amino acid derivatives analyzed on a Chirasil Val column (50 m) connected to a GCQ ion trap mass spectrometer. The constituent amino acids were identified by their characteristic mass spectra, and their chirality was determined where possible by comparison to standard D, L amino acids.

II.6. Growth of strain Bio 33 under different cultivation conditions

With the aim of enhancing the amount of the four active compounds produced by strain Bio 33, we examined the effects of media (BG 11, MBL), the presence/absence of nitrate and cobalt in media BG 11 and MBL, temperature and light on the growth and the production of the desired compounds. The experiments were carried out in batch culture. 0.5% NaCl (5 g/L) was added to all investigated media.

To estimate the yield of biomass and of extracts, strain Bio 33 was cultivated in media BG 11 with and without $NaNO_3$, MBL with and without $NaNO_3$, BG 11 with trace element cobalt and, MBL without trace element cobalt under continuous illumination of 10 μmol photons m^{-2} s^{-1}, room temperature (18 – 20°C) in Fernbach flasks as described in II.1.2.4. The biomass harvested from 7 L medium was extracted and separated by silica gel chromatography (see II.4.2). The main fraction FIII was collected. The extracts and fractions were tested against *Candida maltosa* in agar diffusion assay (see II.3.3). The activity of the extracts and fractions were evaluated based on inhibition zones and MIC values (see II.3.4). The active compounds produced in different media were also analyzed by HPLC with the method developed in II.6.1.

Preparation of the stock culture for the cultivation experiments:

7 mL of a 2 - 3 week old stock culture (stock culture was in the same medium as the test culture) were inoculated into 200 mL Erlenmeyer flasks containing 70 mL of a new fresh medium. After 2 to 3 weeks of incubation at room temperature (18 - 20°C), cyanobacteria were transferred into 2 L Fernbach flasks containing 700 mL medium. The Fernbach flasks were shaken (orbital shaker Innova 2100 Platform Shaker (New Brunswick Scientific USA, USA)) at room temperature at 75 rpm for 2 - 3 weeks and were used as stock cultures for the cultivation experiment. The continuous fluorescent illumination (Radium NL 36W/21 – 840, Spectralux Plus Weiss, Germany) supplied a continuous irradiance of 10 μmol photons m^{-2} s^{-1}. The following cultivation experiments were carried out in the Sartorius incubators (Sartorius AG, Germany) and OSFT/LS/R-32 orbital shaker incubator (TEQ®, Germany) with a shaking speed of 75 rpm. All tested conditions for Bio 33 grew in BG 11 and MBL media are summarized in the following table. ("w/wo" means with and without)

No. \ Factors		Medium	Light intensity (μmol photons m^{-2} s^{-1})	Time of irradiance (h)	Temperature (°C)
1.	Nitrate	BG 11 w/wo nitrate MBL w/wo nitrate	20 20	24 24	18, 22.5, 26 22.5, 26
2.	Cobalt	BG 11 w/wo cobalt MBL w/wo cobalt	20 20	24 24	15, 22.5, 30 15, 22.5, 30
3.	Light intensity	BG 11 w/wo nitrate MBL w/wo nitrate	10, 20	24	22.5
	Time of irradiance		20	12, 24	22.5
4.	Temperature	BG 11	20	24	15, 18, 22.5, 26, 30
		MBL	20	24	15, 22.5, 26, 30

For all experiments, 14 mL suspension of the Fernbach flask were inoculated into 300 mL Erlenmeyer flasks containing 140 mL medium and the flasks were incubated over 4 weeks. All experiments were carried out in parallel. The biomass was harvested every 5 days by Rotanta 460R centrifuge (4100 rpm, 10 min, 20°C), washed with distilled water and lyophilized. The dry weight of biomass was used for estimating the cyanobacterial growth curves in five-day-steps. The dried biomasses were stored at -20°C for later extraction to estimate the production of the active compounds.

The growth of strain Bio 33 cultivated in BG 11 plus 0.5% NaCl at 22.5°C, 20 μmol photons m^{-2} s^{-1}, 12 h/12 h light/dark rhythm was used as reference. Growth was determined by measuring the dry weight of biomass harvested every 5 days and was represented by the growth curves (see above). The specific growth rate constant (μ) of strain Bio 33 under different conditions was estimated based on the expression:

$$\mu = \frac{\ln N - \ln N_o}{t} = 2.303\, x \frac{\log_{10} N_2 - \log_{10} N_1}{t_2 - t_1}$$

where N_2 and N_1 are the biomasses at the different time points (t_1 and t_2) respectively (Fogg *et al.*, 1987).

The mean doubling time G is calculated as following:

$$G = \frac{\ln 2}{\mu}$$

II.6.1. Method development for quantification of the antifungal compounds

The objective of this work was not accurate quantification of balticidins A-C but developing a relative quantification method based on a control sample growing under control conditions to compare the amount of balticidins A-C produced under different cultivation conditions. Thus, method development and optimization for analyzing balticidins A-C from strain Bio 33 based on the dried biomass of strain Bio 33 cultivated in large-scale in a 35 L fermenter aerated with air, with BG 11 medium + 0.5% NaCl, pH 8.5, 22.5°C, light intensity 20 μmol photons m^{-2} s^{-1} under 24 h continuous irradiance, in 35 days (the control biomass).

For quantification experiments, 100 mg biomass were extracted in 50 mL Falcon tubes (VWR, Germany) by stirring at 1000 rpm with a magnetic stirrer (1x0.5cm stirrer bar, Variomag® Electronikrührer Mono H+P Labortechnik GmbH, Germany).

II.6.1.1. *HPLC analysis*

A Shimadzu LC-20a HPLC equipment (see II.4.6.1) was used to perform the method development and validation. LCsolution software was used for data acquisition and system suitability calculations. Different mobile phases such as ACN, MeOH with HCOOH or TFA and different reversed phase columns (see below) were evaluated.

The examined columns are:

1. Synergi 4 μm Hydro-RP 80 Å, 250 x 4.6 mm. (Phenomenex, USA)
2. Luna 5 μm C18 (2) 100 Å, 250 x 4.6 mm. (Phenomenex, USA)
3. LiChrospher 5 μm RP-18e 100 Å, 250 x 4.0 mm. (LiChroCART, Germany)
4. Synergi 4 μm Polar-RP 80 Å, 250 x 4.6 mm. (Phenomenex, USA)
5. Gemini 5 μm C18 (110 Å) 250 × 4.6 mm. (Phenomenex, USA)

II.6.1.2. *Effect of filter and solvent on the preparation of HPLC sample*

The MeOH/H_2O (1:1) extract of strain Bio 33 was used to study the effect of the filters and solvents on the content of the desired compounds. 4 mg of extract were dissolved in 1 mL of solvent MeOH/H_2O in different ratios (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9). The mixture was sonicated for 15 min. Afterwards, the sample was filtered separately by three different filters: cellulose acetate membrane (25 mm, 0.2 μm, VWR, Germany), nylon (25 mm, 0.2 μm, VWR, Germany) and PTFE (12.5 mm, 0.2 μm, VWR, Germany). 20 μL of each sample was analyzed by the HPLC per run.

II.6.1.3. *Effect of solvents on the extraction of balticidins A-C*

100 mg dried biomass was filled into 50 mL Falcon tubes. 45 mL of each solvent (100%, 90%, 80%, 70%, 60%, 50%, 40% MeOH and 100%, 70%, 50% EtOH) were added and the Falcon tubes were sonicated in an ultrasonic bath for 15 min. The biomass was then extracted utilizing magnetic stirrer (1000 rpm) for 24 h. After that, the Falcon was centrifuged (4300 rpm, 7 min, 20°C) and the supernatant was filtered by Whatman filter paper (Grade 1, 11 μm). (This supernatant was marked as E1). The residue was extracted again two times, using 45 mL of the initial extracting solvent and ½ h stirring by magnetic stirrer for each time. (The supernatants were marked as E2, E3). The residue was dried overnight and further extracted with 45 mL MeOH/H_2O (7:3) for 24 h. (The supernatant was marked as E4). All supernatants were dried in vacuum to remove MeOH and lyophilized to get the crude extract.

For HPLC analysis, the crude extract (combining of E1 + E2 or E1 + E2 + E3) from 100 mg biomass was dissolved in 6.6 mL MeOH/H_2O (7:3). (The volume of the solvent was calculated equivalent to

15 mg dried biomass in 1 mL solvent). Due to the small amount of crude extract, extract E3 and E4 were dissolved in 500 μL and 1 mL MeOH/H_2O (7:3), respectively. The mixture was sonicated for 15 min. Afterwards, the samples were filtered with cellulose acetate membrane filters (0.2 μm). 40 μL of each sample was analyzed by HPLC.

II.6.1.4. *Effect of extraction time and volume of extraction solvent*

100 mg dried biomass was filled into a 50 mL Falcon tube. The solvent MeOH/H_2O (7:3, v/v) was added and the Falcon tubes were sonicated for 15 min. The extraction was carried out with magnetic stirrer at 1000 rpm. The solvent volume and the extracting time are shown in Table II-11.

After each extraction, the Falcon was centrifuged (4300 rpm, 7 min, 20^{o}C) and the supernatant was filtered by Whatman filter paper (Grade 1, 11 μm). The supernatants namely E1, E2, E3, E4 were rotary evaporated and lyophilized to get the crude extract. The crude extract of 100 mg biomass was filtered with cellulose acetate membrane filter (0.2 μm). 40 μL of each sample was analyzed by HPLC.

Table II-11. Solvent volume and extraction time used to extract the biomass

No.	Volume of solvent x time of extract x times	Extract
1.	25 mL x 2 h x 3 times	E1+E2+E3
	25 mL x ½ h	E4
2.	35 mL x 2 h x 3 times	E1+E2+E3
	35 mL x ½ h	E4
3.	45 mL x 2 h x 3 times	E1+E2+E3
	45 mL x ½ h	E4
4.	25 mL x 24 h + 25 mL x ½ h	E1+E2
	25 mL x ½ h	E3
5.	35 mL x 24 h + 35 mL x ½ h	E1+E2
	35 mL x ½ h	E3
6.	45 mL x 24h + 45 mL x ½ h	E1+E2
	45 mL x ½ h	E3

II.6.1.5. *Effect of tubes used for extraction on quantification of active compounds*

100 mg dried biomass was extracted with and 45 mL MeOH/H_2O (7:3) by utilizing magnetic stirrer (1000 rpm) for 24 h in two different containers: 1) 100 mL glass Erlenmeyer flask (Merck, Germany) with cellulose steristopper (Fisher Scientific GmbH, Germany) wrapped by aluminum foil; 2) 50 mL

Falcon tube (VWR, Germany). After extraction the tubes were centrifuged (4300 rpm, 7 min, 20°C) and the supernatant was filtered by Whatman filter paper (Grade 1, 11 μm). The residue was reextracted for ½ h. The supernatants were pooled, solvent was removed with rotary evaporator and lyophilized to obtain the crude extract. The samples were filtered with cellulose acetate membrane filters (0.2 μm). 40 μL of each sample was analyzed by HPLC. The experiment was done with two different biomasses and each was repeated 3 times.

II.6.1.6. *Effect of ultrasound and position of the extraction tube*

To estimate the stability of the extract components, the HPLC sample (concentration equivalent to 15 mg biomass/mL solvent) was sonicated for 5, 15, 30, 60 min, 2 h, 6 h and 12 h, respectively, in Falcon standing vertically in water bath. After filtration and removing solvent, samples were analyzed by HPLC. The experiment was done in replicate.

To estimate the effect of the position of the Falcon on the active compounds, 100 mg dried biomass was filled into a 50 mL Falcon tube and 45 mL MeOH/H_2O (7:3) were added. The Falcon tubes were positioned horizontally or vertically in the ultrasonic bath and sonicated for 0, 15, 30, 60 min. The extraction was carried out utilizing magnetic stirrer (1000 rpm) for 24 h. After the first extraction cycle, the tubes were centrifuged (4300 rpm, 7 min, 20°C) and the supernatant was filtered by Whatman filter paper (Grade 1, 11 μm). The residue was reextracted for ½ h. The supernatants were pooled, solvent was removed with rotary evaporator and lyophilized to obtain the crude extract. The samples were filtered with cellulose acetate membrane filters (0.2 μm). 40 μL of each sample was analyzed by HPLC.

II.6.1.7. *Evaluation of the extraction conditions*

100 mg dried biomass was filled into a 50 mL Falcon tube. 45 mL MeOH/H_2O (7:3) were added and the Falcon tube was placed horizontally in the ultrasonic bath for 15 min. The biomass was extracted utilizing magnetic stirrer (1000 rpm) for 24 h. After extracting, the Falcon was centrifuged (4300 rpm, 7 min, 20°C) and the supernatant was filtered with Whatman filter paper (Grade 1, 11 μm). The residue was extracted again three times, each with 45 mL MeOH/H_2O (7:3) for 1/2 h. The supernatants were collected separately, namely E1, E2, E3 and E4, rotary evaporated to remove MeOH and lyophilized to obtain the crude extract.

For analytical HPLC, the crude extract was dissolved in MeOH/H_2O (7:3). The concentration used was equivalent to 15 mg biomass in 1 mL solvent. The Falcon was placed horizontally in the ultrasonic bath and sonicated for 15 min. Before analyzing by HPLC, the sample was filtered through a cellulose acetate membrane filter (0.2 μm). 40 μL of sample was loaded on the LiChrospher RP-18 column

(100 Å, 250 x 4 mm, 5 μm), precolumn C18 (2) with mobile phase MeOH/H_2O plus 0.05% TFA, (solvent gradient program 2, see II.4.6.2) and a flow rate of 1.0 mL min^{-1}. Detection at 226 nm.

II.6.2. Method validation for quantification of the antifungal compounds

The developed method was validated according to the FDA (US FDA, 2001), ICH guidelines (ICH, 1996) and the guidelines comparison according to Chandran (Chandran *et al.*, 2007). The validation included tests on method reproducibility, linearity, accuracy and stability of samples. Biomass of strain Bio 33 cultivated in 35 L fermenter with BG 11 + 0.5% NaCl, pH 8.5, 22.5°C, 24 h continuous irradiance was used to perform the method validation.

II.6.2.1. *Method reproducibility*

Method reproducibility was determined by measuring the repeatability and intermediate (between-days) precision of retention times and peak areas for peaks 1, 2, and 3 (Figure III-29). Repeatability was determined by replicate injection (n = 6) of a 70% MeOH extract of the control biomass with a concentration equivalent to 15 mg biomass in 1 mL solvent (70% MeOH in H_2O). The intermediate precision was also evaluated over two days by performing six successive injections each day.

The relative standard deviation (%RSD) is used to express the precision and repeatability of the assay.

$$\%RSD = \frac{s}{x} \times 100$$

where $s =$ standard deviation of the array; $x =$ mean of the array

II.6.2.2. *Linearity*

Detector response linearity was assessed by preparing seven calibration sample solutions of the control crude extract (concentration equivalent to 15 mg biomass in 1 mL solvent) in the range from 0.75 mg/mL to 15 mg/mL in MeOH/H_2O (7:3). The regression equation was obtained by plotting peak area against concentration, using the least squares method. Linearity was checked in six replicates in the same concentration range using the same stock solution. The %RSD of the slope and Y-intercept of the calibration plot were calculated.

II.6.2.3. *Accuracy and recovery*

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. In our study, accuracy is determined by analysis of known biomass. Three determinations per each concentration in the range of expected concentrations were measured.

The recovery of active compounds in HPLC analysis based on the detector response obtained from each peak extracted from a certain amount of the control biomass. Recovery experiment was performed by comparing the analytical results for extracted samples at low, medium, and high concentration in the range of expected concentrations, each with three replicates.

2, 4, 8, 15, 30, 40, 60 and 100 mg biomass were extracted by the developed method. The crude extracts were dissolved in 2 mL MeOH/H_2O (7:3) and analyzed by HPLC. The linearity of the analytical procedure was checked based on the peak area-responses which are directly proportional to the analyte concentration. These peak areas were applied to the linear regression equation to determine the accuracy of the method. The recovery was calculated from the slope and Y-intercept of the obtained calibration plot.

II.6.2.4. *LOD and LOQ*

The limit of detection (LOD), defined as smallest amount of analyte that can be clearly detected above the baseline signal, was estimated as the amount for which the signal-to-noise ratio was 3. The limit of quantification (LOQ), defined as the lowest concentration of analyte that can be quantified with suitable precision and accuracy, was estimated as the amount for which the signal-to-noise ratio was 10. LOD and LOQ were determined by injecting a series of diluted solutions from the control. LOD and LOQ were also calculated by using the equations LOD = 3.3 x S_{YX}/s and LOQ = 10 x S_{YX}/s, where S_{YX} is residual variance from the regression and s is the slope of the calibration plot.

The precision of the method at the limit of quantification was checked by analysis of five test solutions of the control biomass prepared at the LOQ level and calculating the %RSD of peak area.

II.6.2.5. *Stability in solution*

The stability of balticidins A-C in the crude extract solutions in MeOH/H_2O (7:3) in the autosampler (at 25°C) was evaluated by analyzing one sample with a concentration equivalent to 15 mg biomass/mL three times a day on three different days at a time interval of 7 h. The samples stayed in the autosampler over the period.

II.6.3. Production of bioactive compounds from strain Bio 33

The active compounds were analyzed based on the developed method (described in III.4.1.7). The production of bioactive compounds by strain Bio 33 cultivated in 35 L fermenter with BG 11 medium plus 0.5% NaCl at 22.5°C, 20 μmol photons m^{-2} s^{-1}, under 24 h continuous irradiance was used as control.

II.6.4. Statistical treatment

Student's paired test and one-way ANOVA were used to analyze statistical differences related to growth and antifungal compound analysis. A value of $P < 0.05$ was considered statistically significant. Data were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc., USA). All related data were expressed as mean ± standard deviation (SD) of 2 independent observations. Correlation coefficients between the concentration of balticidins and biomass were calculated for each experiment.

II.7. Isolation and purification of the active compounds from the culture medium of strain TVN40

II.7.1. Extraction of intracellular bioactive compounds

For screening of intracellular bioactive compounds from strain TVN40, the dried biomass was extracted sequentially by *n*-hexane, MeOH and distilled water as shown in Figure II-3.

II.7.2. Extraction of extracellular bioactive compounds

II.7.2.1. *Extraction of the extracellular bioactive compounds with EtOAc*

For antimicrobial screening, the culture medium of TVN40 was extracted as described in II.2.2.2. The aqueous layer was further extracted with dichloromethane.

II.7.2.2. *Extraction of the extracellular bioactive compounds with Amberlite™ XAD-16*

The development of a method using Amberlite XAD-16 to separate the antibacterial fraction out of the culture medium of strain TVN40 was represented in detail in the Master thesis of Heinek (2011). Based on the results of Heinek, the cultivation medium of TVN40 was extracted with an open glass column packed with Amberlite™ XAD-16.

Amberlite™ XAD-16 is hydrophobic polyaromatic resin (dipole moment 0.3) and has a surface area of 900 m^{-2} g^{-1}, a particle size of 0.3 – 1.2 mm, a wet density of 1.02 g mL^{-1}, an average pore diameter of 100 Å, and a pore volume of 1.82 mL g^{-1}.

A suspension of 52 g Amberlite™ XAD-16 in 50% MeOH was poured carefully into a glass column (Ø 5.5 cm) packed by a layer of cotton wool followed by sea sand. On the top of the resin bed, a small layer of glass wool followed by sea sand was applied to protect the resin layer from disturbing when medium and eluent were added. The resin was washed with 500 mL distilled water. A volume of 6.5 L culture medium passed the resin with a flow rate of 1.5 mL min^{-1}. Afterwards, the eluents - H_2O

(650 mL), 80% MeOH (650 mL), 100% MeOH (650 mL), acetone (1300 mL) and dichloromethane (1300 mL) - sequentially passed the column. The elution rate was 13 mL min^{-1}. The elutes were collected as a whole and dried by rotary evaporator lyophilization. The dried extracts were stored at -4°C until use.

II.7.3. Antimicrobial activity

All of the isolated extracts and fractions of strain TVN40 were screened for antimicrobial activity by agar diffusion test as described in II.3.3.

II.7.4. Purification of the active compounds by HPLC

Equipments: see II.4.6.1.

HPLC column: Synergi Hydro RP, 80 Å, 250x4.6 mm, 4 μm (Phenomenex LTD, Germany)

MeOH, ACN: HPLC grade (Merck, Germany)

H_2O: Milli-Q® Advantage A10 ultrapure water purification system (Merck, Germany)

II.7.4.1. *Purification of acetone fraction*

For analytical HPLC, the column Synergi Hydro RP was used with mobile phase ACN/H_2O with a flow rate of 1 mL min^{-1} according to the following HPLC programs. Detection was done at 210, 220, 238, 254, 366 nm.

Program 1:

Time	0.5	18.5	22.5	24.5	26.5
%B	5	60	100	100	5

Program 2:

Time	0.5	18.5	22.5	35	37
%B	5	60	100	100	5

Program 3:

Time	0.5	35	50	53	57
%B	5	100	100	5	5

The successful isolation of pure compounds was carried out with the Synergi Hydro RP column, mobile phase ACN/H_2O (program 4), with a flow rate of 1 mL min^{-1}. Detection was recorded at 210 and 238 nm.

Program 4:

Time	0.5	20.5	25.5	33.5	35
%B	60	90	100	100	60

II.7.4.2. *Purification of 80% methanol fraction*

The Synergi Hydro RP column (80 Å, 250 x 4.6 mm, 4 μm, Phenomenex) was used to analyze the active fraction MeOH80 and other active fractions according to program 1 and program 2, mobile phase ACN/H_2O, flow rate 1 mL min^{-1}. Detection was done at 210, 226, 238, 254 nm by DAD. Sample concentration: 10 μg/20 μL/ injection.

Program 1:

Time	0.5	7.5	20.5	22.5	27.5	29.5	34.5
%B	10	15	16	75	78	100	100

II.7.5. Structural elucidation of isolated compounds

The structural elucidation of the isolated compounds was executed at the Leibnitz institute of Plant Biochemistry in Halle (Germany). All 1D and 2D spectra were recorded on a Varian VNMRS 600 NMR spectrometer operating at 599.832 MHz using a 5 mm inverse detection cryoprobe. Signals were assigned by HMBC and comparison with published data (Suárez-Castillo *et al.*, 2006). The proposed structure is supported by HR-MS. Internal reference: ^{1}H: TMS = 0 ppm; ^{13}C: CD_3OD = 49.0 ppm.

II.7.6. Investigation of growth of strain TVN40

The stock culture, the batch culture and the large scale culture of strain TVN40 were prepared as described in II.1.2.3., II.1.2.4. and II.1.2.5. The large scale cultivation was maintained at 26°C, pH 8.5, and light intensity of 20 μmol photons m^{-2} s^{-1}. The biomass was harvested after 30 days. The medium was stored at 4°C until use. The growth of strain TVN40 in different media (BG 11 medium, BG 11 with Co^{2+}, MBL and MBL with Co^{2+}) was evaluated based on dry weight of biomass over a cultivation time of 30 days. To prepare the stock culture for the investigations, 7 mL of a 2 – 3 week old TVN40 culture were inoculated into 200 mL Erlenmeyer flasks containing 70 mL medium. After 2 to 3 weeks incubation at room temperature (18 - 20°C), cyanobacteria were transferred into 2 L Fernbach flasks containing 700 mL medium. The Fernbach flasks were shaken at room temperature at 75 rpm for 2 - 3 weeks. The continuous illumination by fluorescent lamps (Radium NL 36W/21 – 840, Spectralux Plus Weiss, Germany) supplied a continuous irradiance of 10 μmol photons m^{-2} s^{-1}. For all investigations, 14 mL of the Fernbach flask were inoculated into 300 mL Erlenmeyer flask containing 140 mL medium and the cultivations were carried out in parallel. The biomass was harvested every 5 days by centrifugation (4300 rpm, 10 min at 20°C), washed with distilled water and lyophilized. The dry weight was used for estimating the cyanobacterial growth curves in five-day steps.

II.8. Isolation, cultivation, extration and testing of bacteria from SRC

II.8.1. Materials

- Standard II nutrient agar plate (see Table II-4)
- Malt agar plate (see Table II-5)
- TYG agar plate (see Table II-12)
- Tryptic soy agar (TSA) (Difco ™ Tryptic Soy Agar, Becton, Dickinson & Company, France)
- Luria-Bertani (LB) agar plate (see below)
- 1.5 mL Eppendorf tube (Eppendorf AG, Germany)
- Eppendorf Centrifuge 5430 R (Eppendorf AG, Germany)
- Centrifuge Rotanta 460R (Andreas Hettich GmbH & Co. KG, Germany
- Laminar flow box (Heraeus Instruments, Germany)
- Incubator Mytron BS 120 (Memmert, Germany)
- OSFT/LS/R-32 orbital shaker incubator (TEQ®, Germany)
- Plastic inoculating loop, sterile (VWR, Germany)
- Sterilized L-shape rod (Merck, Germany)
- Micropipettes and tips for 100 - 1000 μL (Carl Roth, Germany)
- NaCl 0.9% (Merck KGaA, Germany)
- Fresh sterilized BG 11 + Cobalt medium (see Table II-2)

Ingredients of TSA:

	Amount (g/L)
Pancreatic Digest of Casein	15.0
Papaic Digest of Soybean	5.0
NaCl	5.0
Agar	15.0
Aquadest	1 L

40 g mixture of TSA was suspended in 1 L distilled water, mixed thoroughly and autoclaved at 121°C for 20 min. To prepare the plate, under laminar flow box, 20 mL of the mixture was poured into a 9 mm Petri dish. After solidification of agar, the plate was stored at room temperature until use.

Ingredients of LB agar plate:

	Origin	Amount (g/L)
Tryptone	Sigma-Aldrich, Germany	10.0
Yeast extract	DIFCO, USA	5.0
NaCl	Carl Roth, Germany	5.0
Bacto agar	Becton, Dickinson & Company, France	12.0
Aquadest		1 L

All components of LB were weighed and suspended in 1 L distiled water, mixed thoroughly and autoclaved at 121°C for 20 min. To prepare the plate, under laminar flow box, 20 mL of the mixture was poured into a 9 mm Petri dish. After solidification of agar, the plate was stored at room temperature until use.

II.8.2. Isolation of bacteria from culture medium of SRC

Dilution method was used to obtain a pure bacterial culture. 5 types of agar plate were used including TSA, TYG, standard NA II agar, Malt and LB. 1 mL of a 20 day-old SRC culture containing in an Eppendorf was sonicated for 3 x 1 minutes. The Eppendorf tube was centrifuged for 30 s at 6500 rpm, 20°C. The supernatant (aliquot A) was diluted to 10^{-5}, 10^{-6} with fresh BG 11 + Co medium. 100 μL of these samples were streaked onto each agar plate using sterilized L-rods. All plates were incubated at 36°C for 3 days except for Malt agar plate at 26°C. The agar plates were stored at 4°C. A single colony of each type of bacteria was diluted to 10^{-5} with BG 11 + Co medium and 100 μL of this sample was pipetted into an agar plate and streaked using a sterilized L-rod. The plate was incubated at 36°C. The dilution process of a single colony was repeated three more times. Isolated bacteria were cultivated separatedly on TYG agar plates and applied for taxonomic identification.

II.8.3. Cultivation of bacteria from culture medium of SRC

The cultivation was carried out with the mixture of bacteria of SRC. Bacterial aliquot A (see II.8.2) was streaked on a TYG agar plate and incubated at 36°C for 2 days. A full loop of bacteria was mixed well in 2 mL of NaCl 0.9%. 18 mL of TYG medium was added. The bacterial concentration of this stock was 2.85×10^6 CFU/mL (OD_{560} = 0.278). 300 μL of the bacterial stock was inoculated in 150 mL fresh TYG medium contained in a 300 mL Erlenmeyer flask. A total of 5 L TYG medium was cultivated with bacteria. After 2 days of cultivation at 33°C in the dark and at 100 rpm, the bacterial biomass and culture medium were separated by centrifugation using 50 mL Falcon tube at 4300 rpm, 10 min, 4°C. The biomass was washed with fresh TYG medium, lyophilized and extracted with *n*-hexane, MeOH and H_2O according to II.2.2.1. The supernatant was shaked with a same amount of EtOAc for 24 h, 2 times. The EtOAc extract was dried with Na_2SO_4. EtOAc was removed by rotary evaporator at 40°C.

As control, 2 L of a double concentration of TYG medium was prepared, autoclaved and was extracted with the same procedure as the culture medium. Antimicrobial activity of all extracts was tested according to II.3.3.

II.8.4. ESI MS analysis

Biomass extracts and EtOAc extracts of culture media of non-axenic TVN40, SRC, bacteria isolated from SRC, and TYG medium were analyzed using a HPLC/MS system - LCMS-8030 Triple Quadrupole Liquid Chromatograph Mass Spectrometer (LC/MS/MS) (Shimadzu, Japan) for the presence of flourensadiol, the dioxindole derivative and other isolated compounds.

Parameter for the HPLC/MS analysis:

- Scan mode in positive and negative mode, with Q1 and Q3

- HPLC system SHIMADZU LC-20a prominence UFLC (SHIMADZU Corporation, Japan)
  - Diode array detector SPD-M20A
  - Auto sampler SIL-10AF
  - Pumps LC-20AD
  - Control unit CBM-20A
  - Column oven CTO-10AS
  - LCsolution software
- HPLC column: Synergi Hydro RP column (80 Å, 250 x 4.6 mm, 4 μm, Phenomenex)
- HPLC gradient:

Time	0.5	12.5	18.5	22.5	24.5	26.5
%B	5	25	60	100	100	5

- Mobile phase (LCMS-grade): A: Water, B: ACN
- Flow of mobile phase: 0.64 mL/min
- Interface temperature: HB+DL each 230°C
- Nitrogen-Flow: Nebulizing gas: 3 L/min, Drying gas: 15 L/min
- Oven-Temperature: 25°C

II.9. Axenization of cyanobacteria

Equipment and materials:

- Laminar flow box (W. H. Mahl Reinraum-, Klima- und Labortechnik, Germany)
- Fresh BG 11 medium normal and BG 11 medium 2x concentration (see II.1.2.1)
- Sterile plastic Petri dishes Ø 9 mm (Carl Roth, Germany)
- Sterile disposable Pasteur pipettes (VWR, Germany)
- Sterile glass Pasteur pipettes 230 mm (VWR, Germany)
- Glass L-shape rod
- Sterile 24-well plate (TPP, Switzerland)
- EtOH (Merck, Germany)
- Microscope (Planapo 1.6x, Leica, Germany)
- Flexible, silicone tube (Rotilabo®-Silicone tube, Carl Roth, Germany):
    - 4.0x1.5x7.0 mm
    - inner Ø 6 mm, Ø outer 9 mm
- 25 mL and 50 mL Erlenmeyer flasks closed with cellulose steristopper, wrapped by aluminum foil and sterilized.
- Cellulose steristopper No. 18d, No. 29 (Fisher Scientific GmbH, Germany)
- Sterile Rotilabo® syringe filter, PVDF, 0.22 μm, Ø 33 mm (Carl Roth, Germany)
- Filter wool (DOHSE Aquaristik KG, Germany)
- Parafilm (Parafilm® "M", USA)
- TYG plate (see below)
- Medium-agar plate (see below)

Table II-12. Composition of TYG

	Origin	Amount (g/L)
BBL™ Trypticase™ peptone	Becton, Dickinson & Company, France	10.0
Yeast extract	DIFCO, USA	1.0
Glucose	Carl Roth, Germany	1.0
NaCl	Carl Roth, Germany	8.0
$CaCl_2.2H_2O$	Carl Roth, Germany	0.3
Bacto agar (1%)	Becton, Dickinson & Company, France	10.0
Aquadest		1 L

All components of the TYG medium were weighed and suspended in 1 L distiled water, mixed thoroughly and autoclaved at 121°C for 20 min. Under the laminar flow box, 20 mL of the aqueous mixture was poured into a Petri dish. After solidification of agar, the plate was stored at room temperature until use.

Test for axenization using TYG plate:

Work was carried out under a laminar box. The culture suspension was dropped onto the surface of a TYG plate and let dry. The plate was incubated at 37°C for 48 h. A clear zone around cyanobacterial filaments on the surface of TYG plate was checked carefully with a binocular.

Preparation of medium-agar plate:

The 2x concentration Bacto agar (1.6% Bacto agar in distilled water) and 2x concentration culture medium were prepared in separated flasks, autoclaved and then mixed together after each has been cooled down to near the gelling temperature (Andersen (2005)). The preparation step was done under the laminar flow box. The Petri dishes filled with the agar mixture (30 mL) were stored at room temperature until use.

II.9.1. Single-cell isolation by micropipette

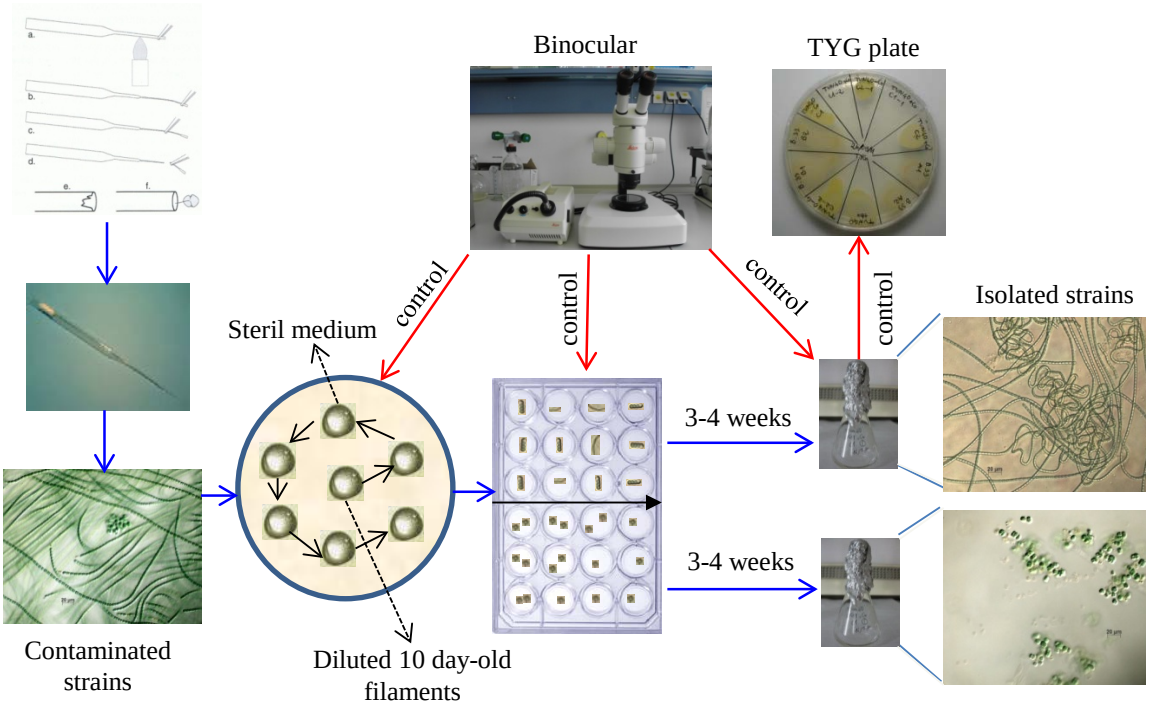


Figure II-7. Isolation of single-cell cyanobacterium by micropipette.

The single-cell isolation was done according to Andersen (2005). 1 mL of cyanobacterial stock culture was inoculated into 10 mL fresh medium in a 25 mL flask. Flasks were incubated at room temperature under continuous illumination (cool white fluorescence tubes, 10 μmol photons m^{-2} s^{-1}). After 2 weeks, the cyanobacteria were checked with Leica binocular. Well-grown cyanobacteria were used for isolation. One drop of the cyanobacterial suspension was dropped into the surface of a sterile Petri dish. 7 - 10 drops of fresh sterile medium were dropped around the drop of cyanobacterial suspension by a sterile Pasteur pipette.

Micropipette isolation was performed with a sterile Pasteur pipette. The Pasteur pipette was heated in a flame, extended and broken to form a capillary. One single filament was picked up by the micropipette and was transfered carefully into the next sterile droplet of fresh medium. The filament was picked up again and transferred to the second sterile droplet. This process was repeated until a single filament, free of all other microorganisms was obtained. The washed filament was transferred to one well of a 24-well-plate which was already filled with 1 mL of sterile medium/well. The 24-well-plate was sealed with parafilm and incubated at room temperature under low light condition (5 μmol photons m^{-2} s^{-1}). The growth of cyanobacteria was checked by the binocular. After 10 days to 3 weeks, cyanobacterial filaments of each well were dropped on a TYG plate to check the axenization. The axenic filaments were transferred into 25 mL flasks containing 10 mL sterile medium. The medium was renewed after 1 month (see Figure II-7).

II.9.2. Streaking of cyanobacterial suspension

1 mL of 2-week stock culture was inoculated into 10 mL fresh medium in a 25 mL flask. Flasks were incubated at 22°C under continuous illumination (cool white fluorescence tubes, 10 μmol photons m^{-2} s^{-1}). After 2 weeks, the cyanobacteria were checked with a binocular. Well-grown cyanobacteria were used for axenization. The culture suspension was diluted to 1:100 and 1:1000 with sterile medium. 100 μL of the diluted suspension was dropped onto the surface of a Bactor agar plate and was spread out with an L-shape rod. The agar plate was incubated at room temperature under low light (5 μmol photons m^{-2} s^{-1}) until cell colonies were observed. The growth of cyanobacteria was checked by a binocular. After 10 days to 3 weeks, the single filaments, without bacteria, were picked up by a sterile micropipette. Each single filament was transferred to one well of a 24-well-plate, which was already filled with 1 mL of sterile medium. The 24-well-plate was incubated at room temperature under low light (5 μmol photons m^{-2} s^{-1}). After 3 weeks, cyanobacterial filaments of each well were dropped on a TYG plate to test for axenization. The axenic filaments were transferred into 25 mL flasks containing 10 mL sterile medium. The medium was renewed after 1 month.

II.9.3. Renewing of isolated cyanobacteria

Strain	Medium
Bio 33	BG 11 - NaCl - Co^{2+}
	BG 11 + NaCl - Co^{2+}
	BG 11 - NaCl + Co^{2+}
	BG 11 + NaCl + Co^{2+}
	MBL
TVN40	BG 11 - Co^{2+}
	BG 11 + Co^{2+}
	MBL
SRC	BG 11 - Co^{2+}
	BG 11 + Co^{2+}
	MBL

+: with; -: without

5 mL of non-axenic isolates of strains Bio 33, TVN40 and SRC were cultivated in 100 mL Erlenmeyer flasks containing 50 mL of different sterile medium (see below) at room temperature under continuous illumination (cool white fluorescence tubes, 10 μmol photons m^{-2} s^{-1}). Cyanobacterial cells in each medium were checked after 4 and 5 weeks of cultivation by naked eye and microscope. Cultures in which cyanobacteria grew well and formed green filaments were used for further cultivation.

II.9.4. Preservation of axenic cultures

For preservation of axenic cyanobacteria, a suspension of 3-week-old axenic culture was dropped on the surface of an agar plate (culture medium supplemented with 0.8% Bacto agar) with a sterile Pasteur pipette. After the medium was evaporated under sterile condition, the agar plate was sealed up by parafilm and incubated at room temperature under low light (5 μmol photons m^{-2} s^{-1}). Color of the cells and the agar surface and the growth of cyanobacteria were checked by naked eye and microscope. After 10 days, cyanobacteria were checked for axenization on TYG agar. The axenic cyanobacteria were renewed after 3 to 4 months.

II.9.5. Cultivation of axenic cyanobacteria

A sterile Pasteur pipette was used to inoculate some filaments of an axenic strain growing on a preservation plate into a 25 mL sterile flask containing 5 mL fresh, sterile medium. After inoculation, the stopper of the flask was wrapped with aluminum foil. All steps were carried out under a laminar box, which was sterilized 30 min with UV light. The culture was incubated 2 – 3 weeks at room temperature under low light (5 μmol photons m^{-2} s^{-1}). Axenization was tested using TYG plate and an axenic culture was used as stock culture (with ratio 1:10 v/v) for a large scale cultivation in Fernbach flasks. The Fernbach flasks were aerated during incubation time of 2 – 3 weeks (see Figure II-2). For harvest, extraction and antimicrobial test see II.1.2.4, II.2.2.1 and II.3, respectively.

II.10. Taxonomy

All solvents, reagents and materials were sterilized before use.

II.10.1. Material and equipments

- Bandelin Sonopuls GM70 sonifier (BANDELIN electronic GmbH & Co. KG, Germany)
- Heating block Blockthermostat BT 200 (Kleinfeld Labortechnik, Germany)
- Eppendorf Centrifuge 5430 R (Eppendorf AG, Germany)
- Centrifuge Rotanta 460R (Andreas Hettich GmbH & Co. KG, Germany)
- Electrophoresis apparatus Minisub-cel GT (Bio-Rad Laboratories, USA)
- MJ Mini® Personal Thermal Cycler (Bio-Rad Laboratories, USA)
- Incubator (Bio-Rad Laboratories, USA)
- Heraeus Pico 17 Microcentrifuge (Thermo Scientific, USA)
- Nanodrop 1000 (PEQLAB Biotechnologie GmbH, Germany)
- ImageJ software (Research Services Branch (RSB), National Institutes of Health (NIH))

Solutions:

STET-Buffer

8% Saccharose (Sigma-Aldrich Chemie GmbH, Germany)

5% Triton X-100 (Sigma-Aldrich Chemie GmbH, Germany)

50 mM EDTA (Roche, Germany)

50 mM Tris-HCl, pH 8.0 (Roche, Germany)

TE-Buffer

50 mM EDTA (Roche, Germany)

50 mM Tris-HCl, pH 8.0 (Roche, Germany)

10% SDS (Sigma-Aldrich Chemie GmbH, Germany)

10 mM Tris-HCl, pH 7.5 (Roche, Germany)

5 M and 1 M NaCl (Carl Roth GmbH + Co. KG, Germany)

70% EtOH (Carl Roth GmbH + Co. KG, Germany)

Isopropanol (Carl Roth GmbH + Co. KG, Germany)

Chloroform/Isoamyl alcohol (IAA) (24:1) (Roti®-C/I for extraction of nucleic acids, Carl Roth, Germany)

Lysozyme 20 mg/mL (QIAGEN GmbH, Germany)

RNAse 1 mg/mL, specific activity ≥ 30 U/mg, Boehringer Mannheim GmbH, Germany)

Agarose NEEO ultra-quality (Carl Roth GmbH + Co. KG, Germany)

Ethidium bromide (Sigma-Aldrich Chemie GmbH, Germany)

0.5 x TBE buffer for DNA electrophoresis: in 1 L distil water contain:

5.4 g Tris base (Carl Roth GmbH + Co. KG, Germany)

2.75 g Boric acid (Sigma-Aldrich Chemie GmbH, Germany)

0.35 g EDTA-Na_2 (Sigma-Aldrich Chemie GmbH, Germany)

DNA molecular weight marker II (Roche, Germany)

DNA molecular weight marker VII (Roche, Germany)

QIAquick Gel Extraction Kit for PCR purification (QIAGEN GmbH, Germany)

QG Buffer (solubilization buffer)

EB Buffer (elution buffer)

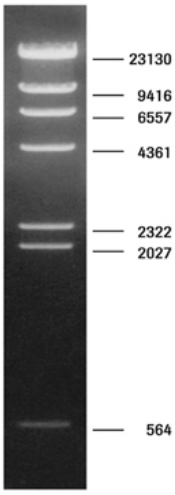
PE Buffer (wash buffer)

II.10.2. Cyanobacterial DNA isolation

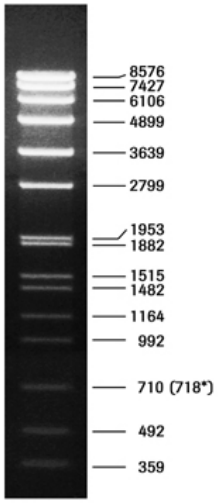
The experiments were done in cooperation with the Pharmaceutical Biotechnology, Institute of Pharmacy, Ernst Moritz Arndt University Greifswald, Germany. The method was modified from a publication of Saha *et al.* (2005).

Axenic cyanobacterium strain Bio 33 was grown in an illuminated shaking incubator at 22.5°C, in medium BG 11 with 0.5% NaCl. Culture was regularly checked for bacterial contamination by plating on Tryptone Glucose Yeast Broth (10.0 g trypticase peptone, 1.0 g yeast extract, 1.0 g glucose, 8.0 g NaCl, 0.3 g $CaCl_2.2H_2O$, 10.0 g Bacto agar in 1 L distile water) solidified medium and incubation overnight at 37°C. Cyanobacterial cells harvested from 50 mL axenic culture (Centrifuge Rotanta 460R, 4100 rpm, 20°C, 2 min) were stored at -20°C until use. Before DNA extraction, the frozen biomass was resuspended in 1 mL TE buffer (50 mM Tris-HCl, 50 mM EDTA, pH 8.0). Cell wall breakage was performed with an ultrasonic probe (30 s, 1 - 3 times). The Eppendorf tube was cooled with ice. After centrifugation (Eppendorf Centrifuge 5430 R, 14000 rpm, 20°C, 10 min), the pellet was resuspended in 300 µL STET-Buffer. 15 µL Chloroform/IAA were added and the sample was moderately shaken for 5 min. 35 µL of the lysozyme were added and the suspension was incubated at 37°C for 1 h. 100 µL 10% SDS and 100 µL 5 M NaCl were added. The suspension was incubated at 37°C for 2 h. 200 µL of 1M NaCl was added and the suspension was incubated at 65°C for 15 min. Extraction of the genomic DNA was performed by adding 750 µL of chloroform/IAA and mixed well.

The suspension was centrifuged at 4000 rpm for 10 min (Eppendorf Centrifuge 5430 R). The upper phase was carefully transferred into a new 1.5 mL Eppendorf tube. The last step (precipitation of protein) was repeated 2 times. One volume of isopropanol was added to the supernatant and the sample was placed in a -20°C freezer for 5 h to precipitate the DNA. After centrifugation (Eppendorf Centrifuge 5430 R, 14000 rpm, 15 min, 4°C), the supernatant was removed. The isolated DNA was washed with 500 µL 70% (v/v) EtOH and centrifuged (Eppendorf Centrifuge 5430 R, 14000 rpm, 15 min, 4°C). The pellet was briefly dried and afterwards resuspended in 20 µL of 10 mM Tris-HCl, pH 7.5. The suspension was warmed up for 5 min at 65°C to swell DNA. RNase treatment was performed at 37°C for 1 h by adding 1 µL of RNAse.



DNA Molecular Weight Marker II (1% agarose gel) (0.12 – 23.1 kbp).



DNA Molecular Weight Marker VII (1% agarose gel) (0.08 – 8.57 kbp).

Figure II-8. DNA Molecular Weight Marker.

The isolated DNA was checked by electrophoresis on 0.6% agarose gel (10 µL ethidium bromide/100 mL gel) in 0.5x TBE buffer. For sample preparation, DNA sample was diluted to 50 – 100 ng/µL with TE buffer. A mixture of 4 µL of diluted DNA solution, 1 µL of 6x loading buffer and 1 µL aqua bidest was mixed on a spot plate and pipetted into one well of the gel. 1 µL of DNA marker II was pipetted into one empty well. The gel was developed with electric source of 90 V, 14 mA and 1 W for 35 min. The bands were measured by ImageJ software.

II.10.3. Polymerase chain reaction (PCR) amplification of the 16S rRNA gene

Purified DNA from Bio 33 and TVN40 isolates was used as the template in a PCR to amplify the 16S rRNA coding regions. Bacterium-specific primers (GM3F and GM4R) (see Table II-14) were used to amplify the 16S rRNA gene. The components of PCR mixtures per 50 µL of reaction mixture are presented in Table II-13. PCR amplifications were performed using an MJ Mini® Personal Thermal Cycler. Denaturation of the DNA was carried out at 95°C for 30 s, while the annealing temperature

was set up at 45°C. Elongation was carried out at 72°C for 1 min 45 s. A total of 35 cycles was carried out (see Table II-15). The PCR products were kept at 4°C.

Table II-13. PCR components.

PCR components	1x (µL)	Origin
10x Polymerase Buffer C	5	
dNTPS (10 mM)	1	
Forward primer GM3F	0.25	
Reverse primer GM4R	0.25	
OptiTaq (5U/µL)	0.25	
Nuclease-free-water	42.75	
DNA Template	0.5	
Sum	50	

Table II-14. Primer sequences and positions (Ansede *et al.*, 2001).

Primer	Positions[a]	Sequence
GM3F[b]	8–24	5'-AGAGTTTGATCMTGGC-3'
GM4R[b]	1492–1507	5'-TACCTTGTTACGACTT-3'

[a]The numbering of the positions is according to that of the 16S rRNA of *E. coli*.

[b]Forward and reverse primers were used to amplify almost the entire 16S rRNA gene from chromosomal DNA.

Table II-15. PCR program.

No.	Step	Temperature (°C)	Time	
1.	Initial denaturing	95	5 m	
2.	Denaturing	95	30 s	step 2, 3, 4 were repeated 34 times
3.	Annealing	45	30 s	
4.	Elongation	72	1 m 45 s	
5.	Final Elongation	72	7 m	
6.	Hold	10	infinite	

To purify the amplified 16S rDNA from PCR products, 2 x 75 µL of PCR products were loaded on a 1.5% agarose gel (w/v) in TBE buffer in comparison with 2 µL of DNA molecular weight marker VII. The desired bands were cut and transferred into an Eppendorf tube. Products were then purified using

the QIAquick Gel Extraction Kit for PCR purification due to the manufacturer's instructions. The 16S rDNA concentration was measured using a Nanodrop 1000 machine.

II.10.4. Phylogenetic relationship

The nearly complete 16S rRNA gene of strain Bio 33 was amplified in a PCR using the universal bacterial primer pair GM3F/GM4R (Muyzer *et al.*, 1995). The amplicon was sequenced by Eurofins MWG Operon (Ebersberg, Germany) using primers GM1F (Muyzer *et al.*, 1993), 907RM (Muyzer *et al.*, 1995) and their reverse complements GM1R/907FK. Single sequencing reads were assembled in Geneious version 6.0.3 created by Biomatters (available from http://www.geneious.com). The 16S rDNA sequence of Bio 33 and related sequences not yet available in the SILVA database were automatically aligned according to the SILVA SSURef NR 111 database (available from http://www.arb-silva.de) using the Silva INcremental Aligner (SINA) version 1.2.11 (Pruesse *et al.*, 2012). Sequences were imported into the ARB software package version 5.3 (Ludwig *et al.*, 2004) and the alignment was manually refined taking into account the secondary structure information of the rRNA. Phylogenetic reconstruction was performed with 46 sequences using a maximum likelihood method. The final tree was calculated with RAxML version 7.4.2 (GTRGAMMA model) (Stamatakis, 2006) and based on 559 distinct alignment patterns.

Numbers given on the branches display bootstrap proportions as percentage of 1000 replicates for values greater than or equal to 50%. *B. subtilis* (AJ276351) and *E. coli* (DQ360844)) sequences were used as outgroup.

III. Results

III.1. Screening for antifungal compounds

III.1.1. Yield of biomass

To prepare the biomass for the screening, cyanobacteria were cultured in batch scale (see II.1.2.4). The dry weight of harvested biomasses is shown in Figure III-1. The highest biomass (2.92 g) was harvested from strain 3 and the lowest biomass (0.91 g) from strain 5. In general, an average of 1.8 g dried biomass was obtained from 7 L cyanobacterial culture after 35 days of cultivation. Some strains were cultivated in different media, such as MBL, MBL without nitrate (MBL – N), BG 11, BG 11 without nitrate (BG 11 – N), BG 11 with cobalt (BG 11 + Co), to study the effect of medium on the amount of biomass. The result recorded from strain 5 and strain 14 showed that cyanobacteria produced more biomass when growing in BG 11 medium.

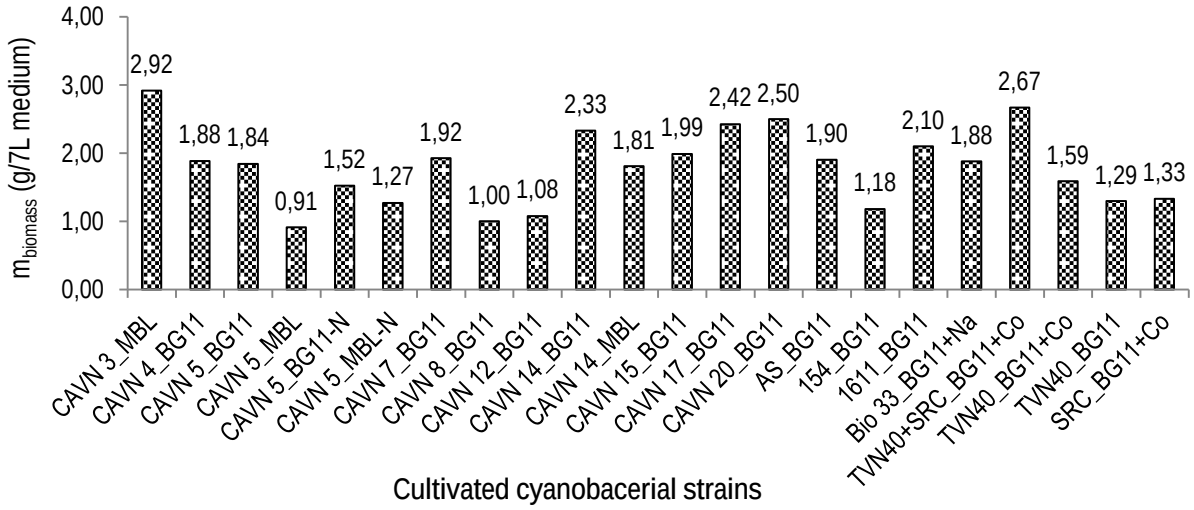


Figure III-1. The yield of biomass of cultivated cyanobacteria

III.1.2. Yield of the extracts

To prepare the extracts for antimicrobial testing, the dried biomass was extracted with *n*-hexane, MeOH and H_2O (see II.2.2.1). The yield of extraction is summarized in Table III-1. The yield of *n*-hexane, MeOH, and water extracts ranged from 0.18 to 4.36%, 6.82 to 35.64% and 1.66 to 24.36% of the dry weight of biomass, respectively. The EtOAc extract obtained from 3 L culture medium ranged from 3.87 to 14.37 mg. In summary, the methanol extract yielded the highest extract amount while the *n*-hexane and EtOAc extracts yielded only low amounts.

Table III-1. Yield of *n*-hexane, MeOH and H_2O extracts

No	Strain	*n*-hexane (%)	MeOH (%)	H_2O (%)	EtOAc extract (mg/3 L medium)
1.	CAVN 1_BG11	0.44	16.77	7.26	-
2.	CAVN2_BG11	0.35	15.36	8.89	-
3.	CAVN 3_M BL	0.30	6.82	6.01	3.87
4.	CAVN 4_BG11	1.18	11.06	9.18	7.46
5.	CAVN 5_BG11_old	0.18	31.89	13.28	-
6.	CAVN 5_BG11_new	0.70	15.28	1.66	8.07
7.	CAVN 5_MBL	2.13	13.64	14.75	4.32
8.	CAVN 5_BG11-N	2.63	12.25	10.78	7.67
9.	CAVN 5_MBL-N	1.96	14.14	9.37	5.06
10.	CAVN 7_BG11	3.80	11.87	4.21	12.68
11.	CAVN 8_BG11	4.36	12.04	10.02	14.37
12.	CAVN 10_BG11	3.70	22.64	15.68	-
13.	CAVN 11 LH_BG11	0.28	15.08	7.44	-
14.	CAVN 11_BG11	1.32	19.52	6.84	-
15.	CAVN 12_BG11	0.80	35.64	24.36	9.35
16.	CAVN 14_BG11_old	1.16	21.80	8.60	-
17.	CAVN 14_BG11_new	1.30	12.63	6.72	7.88
18.	CAVN 14_ MBL	1.43	10.74	-	8.34
19.	CAVN 15_BG11	0.88	34.50	13.16	8.90
20.	CAVN 16_BG11	0.92	13.86	9.26	-
21.	CAVN 17_BG11	0.54	17.26	11.80	8.89
22.	CAVN 20_BG11	-	12.59	11.39	7.87
23.	233_BG11	2.31	18.13	2.92	-
24.	1_BG11	3.39	13.29	2.55	-
25.	AS_BG11	3.07	29.30	3.69	5.80
26.	154_BG11	1.48	28.77	4.95	4.96
27.	1611_BG11	2.89	18.36	5.35	6.78
28.	Bio 33_BG11+Na_old	2.96	20.67	7.58	8.24
29.	TVN40+SRC_BG11+Co	1.94	23.07	3.38	10.20

III.1.3. Antifungal activity against *Candida maltosa*

133 intra- and extracellular extracts (including 36 *n*-hexane extracts, 43 MeOH extracts, 35 H_2O extracts and 19 EtOAc extracts) from 28 cyanobacterial strains were tested against *Candida maltosa* SBUG 700 using agar diffusion method to screen for antifungal activity. According to the inhibition zones, the antifungal activity of extracts was classified into four levels: low activity (IZ = 7-10 mm),

moderate activity (IZ = 11-15 mm), strong activity (IZ = 16-20 mm) and very strong activity (IZ > 20 mm).

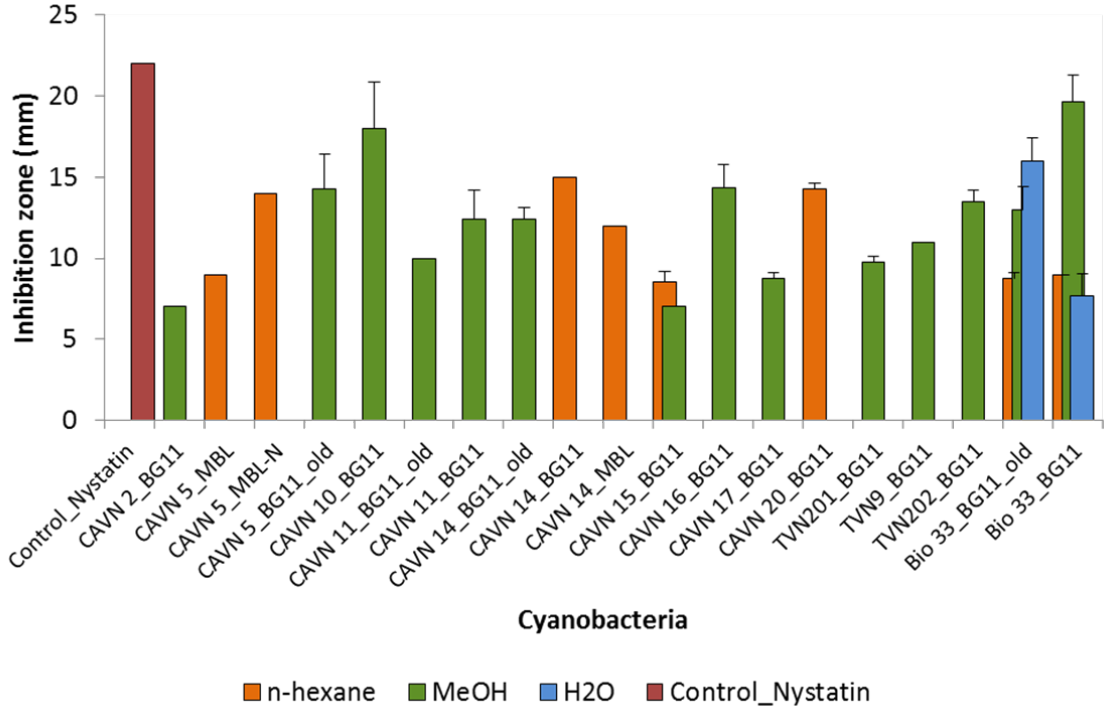


Figure III-2. Antifungal activity of *n*-hexane, methanol and water extracts against *Candida maltosa* SBUG 700.

Agar diffusion assay, n = 3, two parallels, extract concentration 2 mg/6 mm paper disc, inhibition zone including the diameter of paper disc. Concentration of nystatin control was 10 μg/paper disc. “Old” means the biomass was harvested before 2006. Other biomasses were harvested from 2009 - 2010.

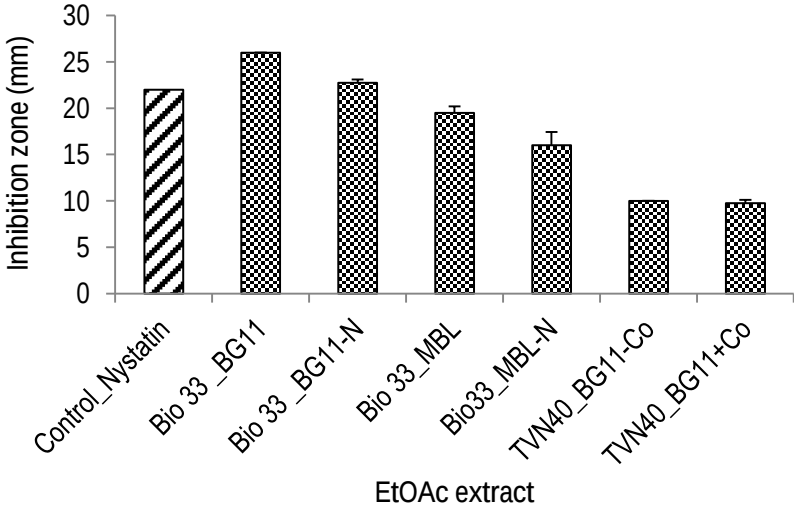


Figure III-3. Antifungal activity of EtOAc extracts against *Candida maltosa* SBUG 700.

Agar difussion assay, n = 2; extract concentration 2 mg/6 mm paper disc, inhibition zone including the diameter of paper disc. Concentration of nystatin control was 10 μg/paper disc).

Extracts exhibiting antifungal activity are shown in Figure III-2 and Figure III-3. Among the tested extracts, 8 *n*-hexane extracts, 14 MeOH extracts, 2 H_2O extracts and 6 EtOAc extracts exhibited activity against *Candida maltosa*, mostly ranging from low to moderate activity. Only the MeOH extracts of Bio 33 and CAVN 10 have shown strong activity. All paper discs loaded only with the solvents showed no inhibitory effects to growth of the yeast.

All extracts prepared from biomass and cultured medium of Bio 33 showed activity against *Candida maltosa*. The MeOH extracts of CAVN 5 and CAVN 14, which were cultivated before year 2006 (named "old" in Figure III-2) showed considerable antifungal activity. Therefore, these strains were cultivated with different media and tested again to confirm the activity. However, antifungal tests of the MeOH extracts from freshly cultivated CAVN 5 and CAVN 14 showed no inhibition zone against *Candida maltosa* although the *n*-hexane extracts showed activity. Only the antifungal activity of strain Bio 33 was confirmed for all culture media.

III.1.4. Antibacterial activity

The antibacterial activity of extracts was classified into four levels: low activity (IZ = 7-10 mm), moderate activity (IZ = 11-15 mm), strong activity (IZ = 16-20 mm) and very strong activity (IZ > 20 mm).

III.1.4.1. *Agar diffusion assay against Bacillus subtilis*

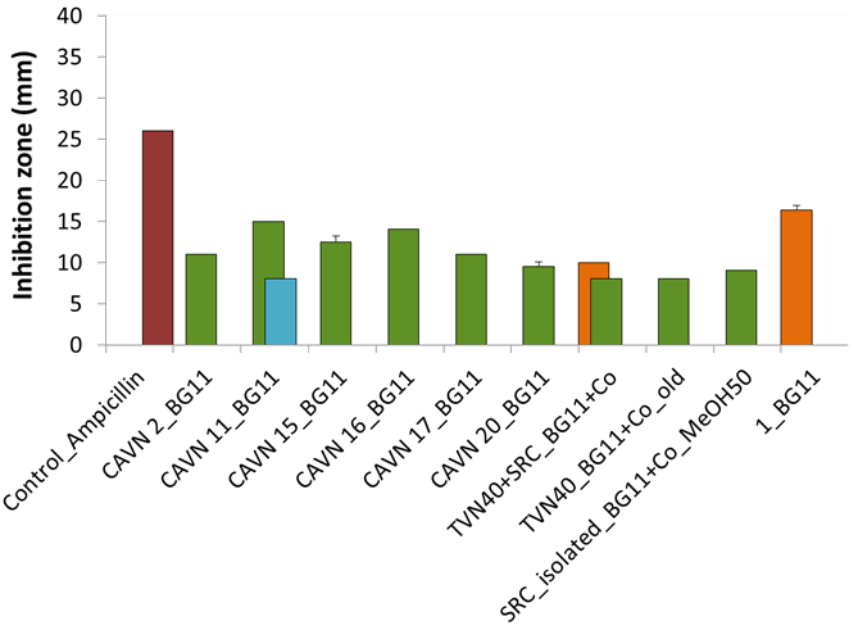


Figure III-4. Antibacterial activity of *n*-hexane, methanol and water extracts against *Bacillus subtilis*.

Agar diffusion assay, n = 3, 2 parallels; extract concentration 2 mg/6 mm paper disc, inhibition zone including the diameter of paper disc. Concentration of ampicillin control was 10 μg/paper disc. "Old" means the biomass was harvested before 2006. Other biomasses were harvested from 2009 - 2010.

The results of agar diffusion assay of the active extracts against *Bacillus subtilis* are shown in Figure III-4. There are nine MeOH extracts displayed low to moderate activity against *Bacillus subtilis* according to the definition before (see III.1.3). Among the tested *n*-hexane extracts and water extracts, only the *n*-hexane extract of strain 1 and TVN40 (mixed culture of filamentous cyanobacteria and SRC) and the water extract of CAVN 11 were active against *Bacillus subtilis* with inhibition zones of 16, 10 and 8 mm, respectively.

III.1.4.2. *Agar diffusion assay against Staphylococcus aureus*

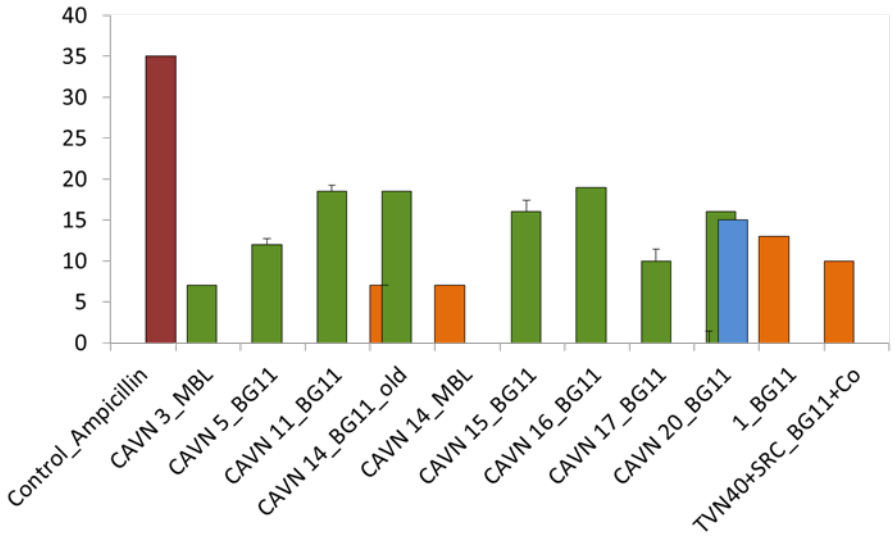


Figure III-5. Antibacterial activity of *n*-hexane, methanol and water extracts against *Staphylococcus aureus.*

Agar diffusion assay, n = 3, 2 parallels; extract concentration 2 mg/6 mm paper disc, inhibition zone including the diameter of paper disc. Concentration of ampicillin control was 10 µg/paper disc. "Old" means the biomass was harvested before 2006. Other biomasses were harvested from 2009 - 2010.

The MeOH extracts of strain 11, 14, 15, 16 and 20 showed strong antimicrobial activity against *Staphylococcus aureus* according to the definition above (see III.1.3). The strongest inhibition was observed at methanol extracts of CAVN 16, CAVN 14 and CAVN 11. The presence of Hapalindolinone A in CAVN 16 (Bui, 2006) and fatty acids (such as myristoleic acid, myristic acid, palmitic acid, linoleic acid) in CAVN 11 may be responsible for the antibacterial activity of these cyanobacteria (Pham, 2007).

III.1.4.3. *Agar diffusion assay against Escherichia coli*

All tested crude extracts showed no inhibition zone against *Escherichia coli*. Ampicillin used as control showed an inhibition zone of 20 mm (15 µg/paper disc).

III.1.4.4. *Agar diffusion assay against Pseudomonas aeruginosa*

Among all of the tested crude extracts, only the MeOH extract of strain TVN40 showed weak activity (inhibition zone 8 mm) against *Pseudomonas aeruginosa*. Gentamicin used as control showed an inhibition zone of 26 mm (25 μg/paper disc).

In summary, all extracts prepared from biomass and cultured medium of strain Bio 33 showed activity against *Candida maltosa*. In addition, in agar diffusion assay all extracts of Bio 33 showed no antibacterial activity. These results indicated specific activity of the methanolic extract of strain Bio 33 towards fungi. It is worth mentioning that the biomass extracts of strain TVN40 (2 mg/6 mm paper disc) showed no inhibition zone against *Candida maltosa*, but the EtOAc extract of this strain was specific antifungal active. Consequently, strain Bio 33 and TVN40 have been chosen for isolation and identification of the active substances.

III.2. Isolation and purification of the antifungal compounds from strain Bio 33

The biomass used for isolation of the antifungal compounds from strain Bio 33 was cultivated 35 days in the big column containing 35 L BG 11 medium plus 0.5% NaCl, 22.5°C, under continuous light with light intensity 20 μmol photons m^{-2} s^{-1}. The culture was aerated with a mixture of air and CO_2 to maintain a pH of 8.5 (see II.1.2.5).

III.2.1. Extraction of the biomass with *n*-hexane, MeOH/H_2O, H_2O

The MeOH and H_2O extracts of strain Bio 33 showed activity against *Candida maltosa* (Table III-2). This fact suggested that the active compounds from this strain are rather polar, so that the extraction of biomass with MeOH was not sufficient to concentrate the antifungal activity in the methanol extract. Further extractions carried out with MeOH and MeOH/H_2O (1:1) confirmed that the antifungal active compounds were extracted better from the biomass by MeOH/H_2O (1:1). The yield of the extraction is presented in Table III-2.

Table III-2. Mass and inhibition zone of the crude extracts from biomass of strain Bio 33.

Extract Test 1	%	IZ (mm)	Extract Test 2	%	IZ (mm)
n-hexane	3.0 ± 0.9	-	*n*-hexane	3.3 ± 1.7	-
MeOH/H_2O (1:1)	30.3 ± 2.3	22.0	MeOH 100%	20.2 ± 1.1	12.0
H_2O	7.7 ± 2.6	-	H_2O	9.8 ± 1.7	16.0

n = 3, 3 parallels

Besides, two extraction methods were evaluated with the same amount of biomass and over the same period of time: 1) Shaking the biomass with a shaker (120 rpm), HS250 basic shaker, KIKA Labortechnik, Germany), 2) Stirring the biomass with a magnetic stirrer (1000 rpm, HeidolpH MR 3000 magnetic stirrer, Heidolph Instruments GmbH & Co. KG, Germany). No significant differences between the amounts of the crude extract were found. The stirring method yielded a 2% higher yield of the crude extract and was chosen for extracting the biomass of strain Bio 33.

III.2.2. Cytotoxicity of the crude extract

The MeOH/H_2O extract of Bio 33 was screened for cytotoxic activity with the 5637 cell line in the range of 1.56 to 100 μg/mL. The extract concentration which inhibited growth of cells by 50% per well (IC_{50}) was calculated (Appendix I). IC_{50} value of the crude extract MeOH/H_2O (1:1) was estimated with 165 μg/mL. Acording to the US National Cancer institute's criteria, an extract is judged as active if the IC_{50} value is below 20 μg/mL, marginal activity (20 μg/mL < IC_{50} ≤ 500 μg/ml), and no activity (IC_{50} > 500 μg/mL) (Suffness *et al.*, 1991), so that only marginal cytotoxicity and no antibacterial activity were observed. These results confirmed the specific antifungal activity of the MeOH/H_2O extract of strain Bio 33.

III.2.3. Analytical thin layer chromatography (TLC) of the crude extract

The MeOH/H_2O (1:1) extract was separated by TLC with different stationary phases (silica gel normal phase and -reversed phase, cellulose, polyamide) as well as mobile systems to identify the separation conditions for column chromatography. Furthermore, the antifungal fraction was localized on the chromatogram by bioautographic assay. From the analytical TCL, silica gel was identified as the best stationary phase for separation the components of the MeOH/H_2O extract.

Figure III-6 show chromatograms developed with the solvent system EtOAc/MeOH/H_2O 7:2:1, 1:7:2 and MeOH/H_2O 8:2 and detected by visible light, UV light 365 and 254 nm and by spraying with anisaldehyde/sulfuric acid reagent showed 17, 10 and 9 separated bands, respectively. There was always one brown band retained at the start line of the TLC sheet.

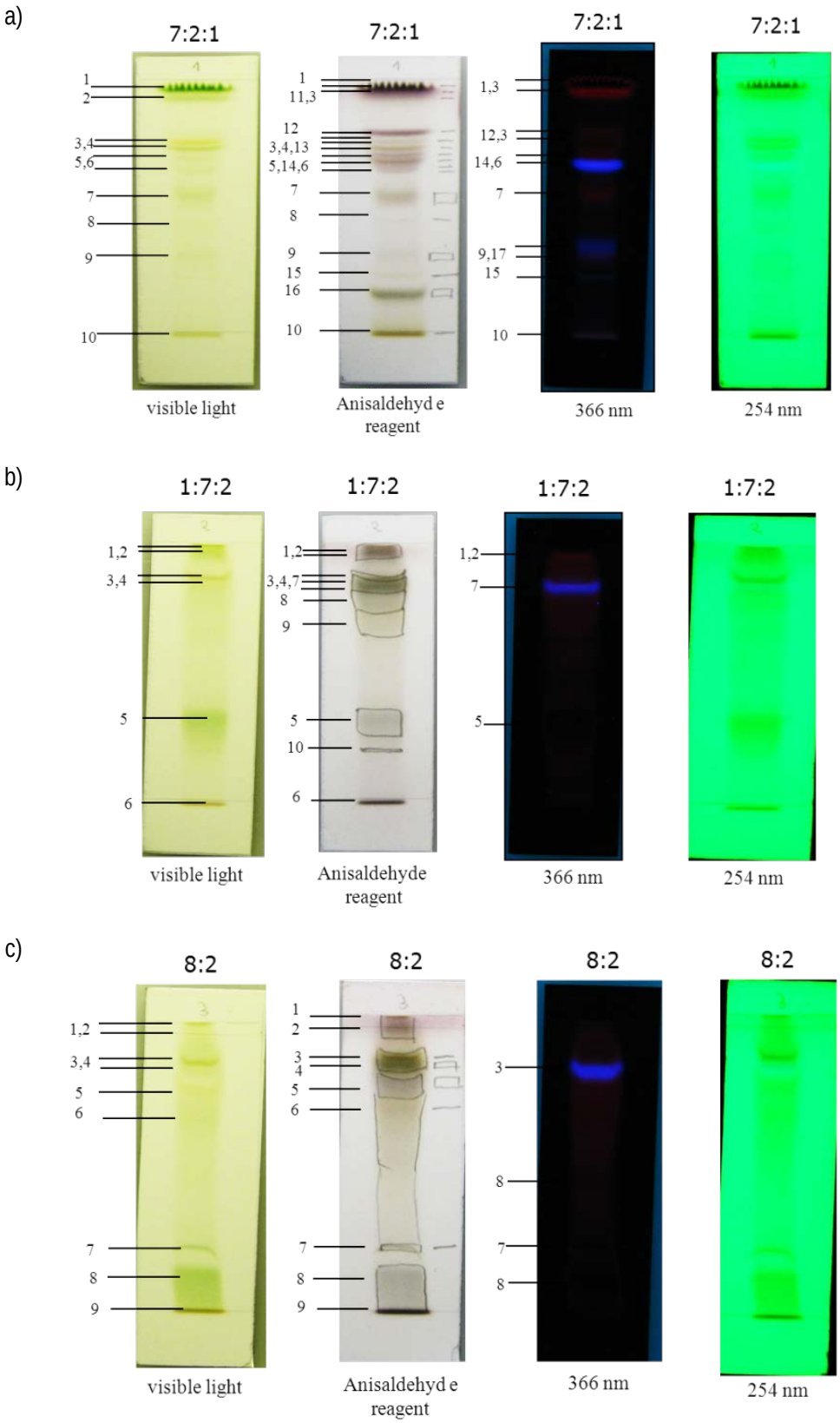


Figure III-6. Thin layer chromatography of the MeOH/H_2O extract from strain Bio 33.

TLCs developed in solvent system: a) EtOAc/MeOH/H_2O (7:2:1). b) EtOAc/MeOH/H_2O (1:7:2). c) MeOH/H_2O (8:2). Detection visible light, UV light 365 nm, 254 nm and anisaldehyde reagent

III.2.4. Autobiographic assay of the crude extract

In the autobiographic test, the TLC developed in EtOAc/MeOH/H_2O (7:2:1) showed an inhibition zone surrounding the start line. This result in combination with the fact that the crude extract was separated in 17 bands demonstrated that the solvent system could not elute the active compounds from the start line but most of inactive and more unpolar components were eluted by this mixture. Hence, this solvent system was usable as initial solvent for silica gel column chromatography to wash out more unpolar compounds.

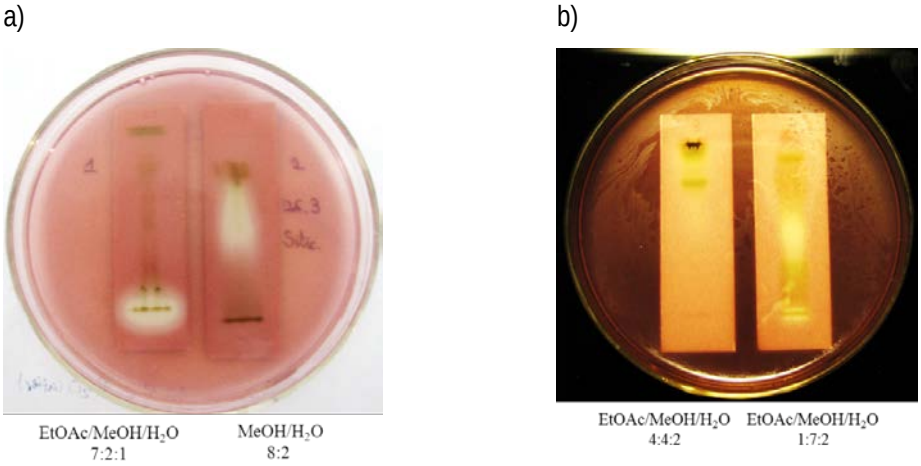


Figure III-7. Autobiographic assay of the MeOH/H_2O extract from strain Bio 33.

The TLC developed on a silica gel stationary phase with a) EtOAc/MeOH/H_2O (7:2:1) and MeOH/H_2O (8:2). b) EtOAc/MeOH/H_2O (4:4:2) and (1:7:2).

The development of the TLC with MeOH/H_2O (8:2), with EtOAc/MeOH/H_2O (4:4:2) and (1:7:2) showed that the active compounds are more polar and migrate better to the front with solvent mixtures with higher polarity, but the separation from the inactive substances was not sufficient (Figure III-7).

From all of the studied solvent systems, the following solvent systems were selected for column chromatography. EtOAc/MeOH/H_2O (7:2:1) was used to wash out the inactive unpolar compounds; followed by more polar mixture of these solvents EtOAc/MeOH/H_2O (5:3.5:1.5) and (1:7:2) to elute the active compounds. At the end, the polar rest was washed out from the silica gel by MeOH/H_2O (5:5).

III.2.5. Fractionation of the MeOH/H_2O extract by silica gel column chromatography

Based on the solvent systems established by bioautography, the column chromatography was carried out with silica gel (0.040-0.063 mm) as stationary phase. For separating 600 mg MeOH/H_2O (1:1) crude extract, the optimized volume of mobile phases and the yield of the fractionation of MeOH/H_2O extract by silica gel are shown in the following table.

Table III-3. The yield of the fractionation of MeOH/H_2O extract by silica gel

Fraction	Volume (mL)	Solvent system	Solvent	Ration	%
FI	200	SS1	EtOAc/MeOH/H_2O	7:2:1	7.12±0.10
FII	150	SS2	EtOAc/MeOH/H_2O	5:3.5:1.5	54.94±4.23
FIII	500	SS3	EtOAc/MeOH/H_2O	1:7:2	26.70±3.07
FIV	400	SS4	EtOAc/MeOH/H_2O	5:5	8.78±2.06

The fraction FII was separated into 3 fractions by TLC with mobile phase EtOAc/MeOH/H_2O (5:3.5:1.5).

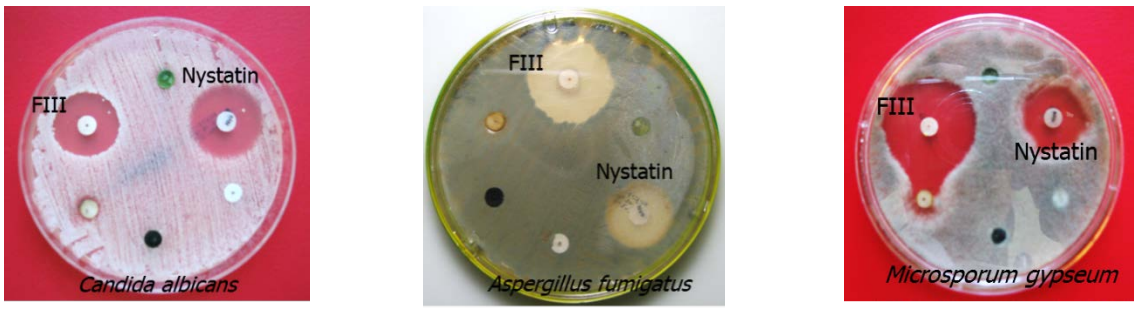
Fraction	%
FII-1	5.26±1.22
FII-2	34.35±2.74
FII-3	15.33±1.46

All fractions FI, FII-1, FII-2, FII-3, FIII and FIV were tested against *Candida maltosa* in a concentration of 500 μg/6 mm paper disc. Only FII-3 and FIII showed activity (IZ = 10 mm and 16 mm, respectively) and were combined for further isolation of the antifungal compounds.

III.2.6. The biological activity of the main fraction FIII

III.2.6.1. *Antifungal activity of FIII*

The main fraction FIII of strain Bio 33 was tested against other pathogenous fungi and yeasts. The results (Figure III-8) confirmed the strong, specific and broad spectrum activity of the active compounds against human pathogenous fungi and yeasts.



Agar diffusion assay, n = 2, extract concentration 1 mg/6 mm paper disc, inhibition zone including the diameter of paper disc.

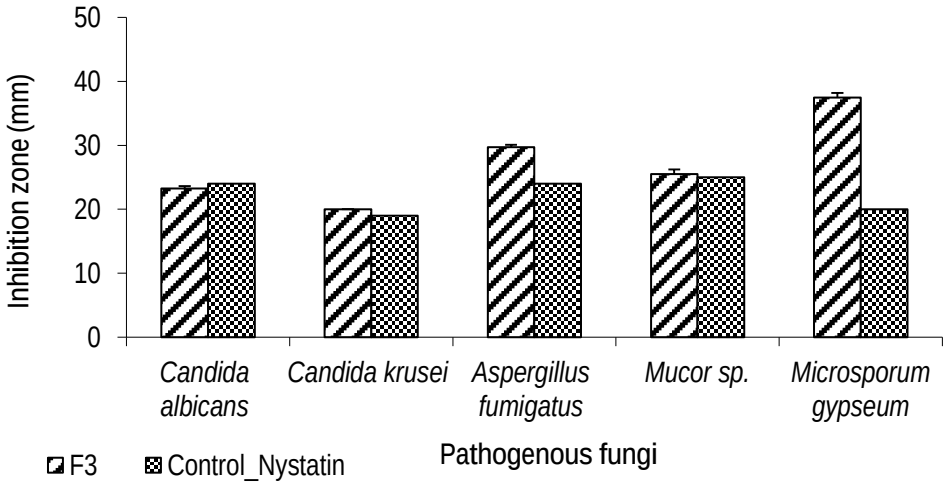


Figure III-8. Antifungal activity of the main fraction FIII against some pathogenous fungi and yeasts.

III.2.6.2. *MIC of FIII against Candida maltosa*

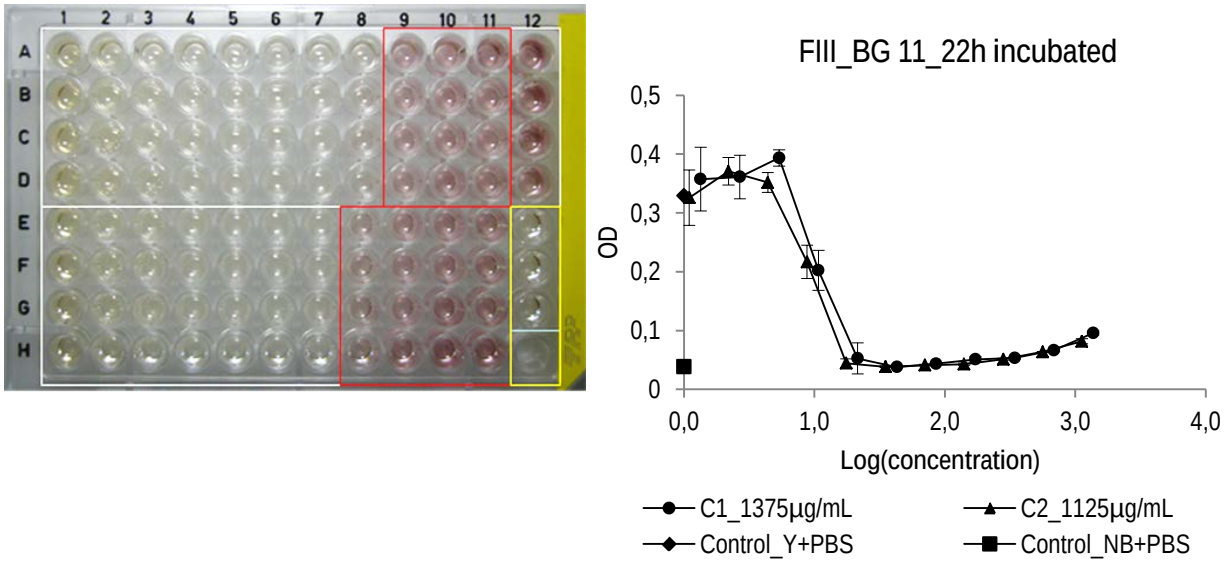


Figure III-9. MIC assay of the main fraction FIII against *Candida maltosa*.

C1 was tested in rows A-D. C2 was tested in rows E-H. Negative control was A12-D12 with PBS + Bouillon + *C. m*. Medium control was in E12-G12 with PBS + Bouillon. H12 is blank. Bars represent the standard deviation.

The main fraction FIII received from column chromatography displayed the MIC value against *Candida maltosa* of 14.2 µg/mL.

III.2.6.3. *Cytotoxicity of FIII*

The main fraction FIII of Bio 33 was tested for cytotoxic activity with the 5637 cell line in the range of 1.56 to 100 μg/mL. The inhibition of cell growth increased when the concentration of the active extracts increased. The concentration of FIII which inhibited growth of cells by 50% (IC_{50}) was estimated as 93 μg/mL, showing marginal cytotoxic activity (see III.2.2 and Appendix I)

III.2.6.4. *Autobiographic assay of FIII*

a) b)

Figure III-10. Autobiographic assay of the main fraction FIII.

The TLC was developed on a silica gel stationary phase with a) EtOAc/MeOH/H_2O (5:3.5:1.5), b) EtOAc/MeOH/H_2O (1:7:2). The control TLC was detected by ninhydrin reagent.

The main fraction FIII was developed with EtOAc/MeOH/H_2O (5:3.5:1.5) and (1:7:2) on silica gel plate. Autobiographic assay showed a clear and stretched activity zone against *Candida maltosa* in both cases.In comparison to the control TLCs (Figure III-10, TLC plates on the left), a migration of the active compounds was observed but stretching from the start line to the front. The spots could not be detected by anisaldehyde reagent or UV light but displayed pink colour with ninhydrin reagent.

From the TLC developed in EtOAc/MeOH/H_2O (5:3.5:1.5) and sprayed with ninhydrin, R_f values were calculated as 0.06, 0.20, 0.33, 0.44 for four bands. This TLC could imply that there are at least four active compounds in the crude extract but they have not been separated completely. Further efforts to separate this fraction by TLC and column chromatography were not successful.

III.2.7. Purification of FIII by solid phase extraction (SPE)

The main fraction FIII (4 mg/mL) was analyzed by analytical HPLC with DAD detector using a reversed phase Gemini C18 column. Four main peaks FIII-4, FIII-5, FIII-6 and FIII-7 (Figure III-11) were separated with a gradient of methanol in water plus 0.05% TFA (HPLC program 2, see II.4.6.2).

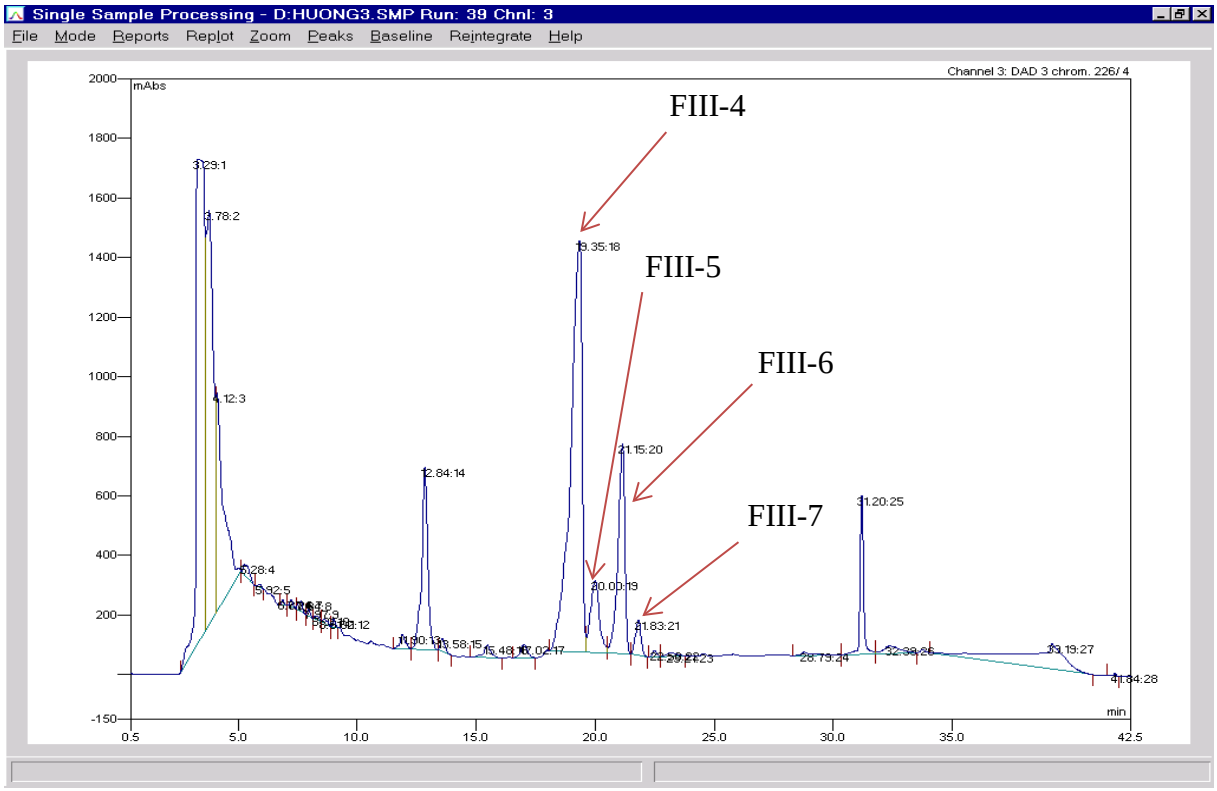


Figure III-11. HPLC of the main fraction FIII.

HPLC conditions: KONTRON Instruments. Column RP Gemini C18 (110 Å, 250 x 4.6 mm, 5 μm, Phenomenex, USA). Mobile phase: MeOH/H_2O 0.05% TFA. Flow rate: 1.0 mL min^{-1}. Detection wavelength: 226 nm. Running program 2. Sample: FIII, concentration 4 mg/mL, injection 20 μL/run.

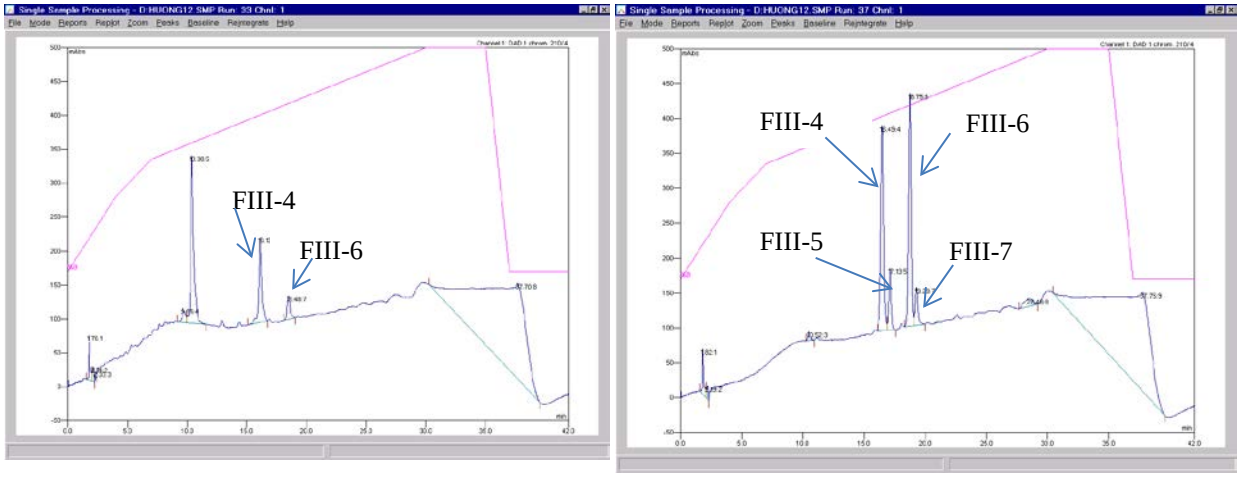


Figure III-12. HPLC spectra of SPE fractions.

HPLC conditions: KONTRON Instruments. Column LiChrospher RP-18e (100 Å, 250x4 mm, 5 μm). Mobile phase: MeOH/H_2O 0.05% TFA. Flow rate: 1.0 mL min^{-1}. Detection wavelength: 226 nm. HPLC program 2. a) Fraction SPE 45%. b) Fraction SPE 80%. Concentration: 3 mg/mL. Injection: 20 μL. HPLC program 2.

To remove the more polar components of fraction FIII (Figure III-11), the SPE technique with a C18 cartridge was used. The SPE cartridge was successively eluted with 5%, 45%, and 80% MeOH in H_2O followed by 100% MeOH. All SPE fractions were tested against *Candida maltosa* in a concentration of 500 μg/6 mm paper disc. Only fraction 45% and 80% MeOH/H_2O showed activity (IZ = 18 mm and 22

mm, respectively). Therefore, fraction 45% and 80% MeOH/H_2O were further purified by reversed phase C18 HPLC to isolate the active compounds.

HPLC-DAD analysis indicated that four main peaks, namely FIII-4, FIII-5, FIII-6, and FIII-7 (Figure III-12), were present in the 45% and 80% MeOH/H_2O fractions. The 45% MeOH/H_2O fraction contained predominantly FIII-4 and FIII-6. The 80% MeOH/H_2O fraction contained all four peaks.

Table III-4. Yield of the purification of the main fraction FIII by SPE

SPE fraction	Volume (mL)	%
After loaded*		58.66±0.23
SPE 5%	100	19.33±0.54
SPE 45%	100	14.93±0.05
SPE 80%	100	6.34±0.03
SPE 100%	100	0.08±0.01

*After loaded: the outflow of loading sample was collected and lyophilized to record the amount of the compounds which were not caught by the SPE cartridge.

III.2.8. Purification of the active compounds by HPLC

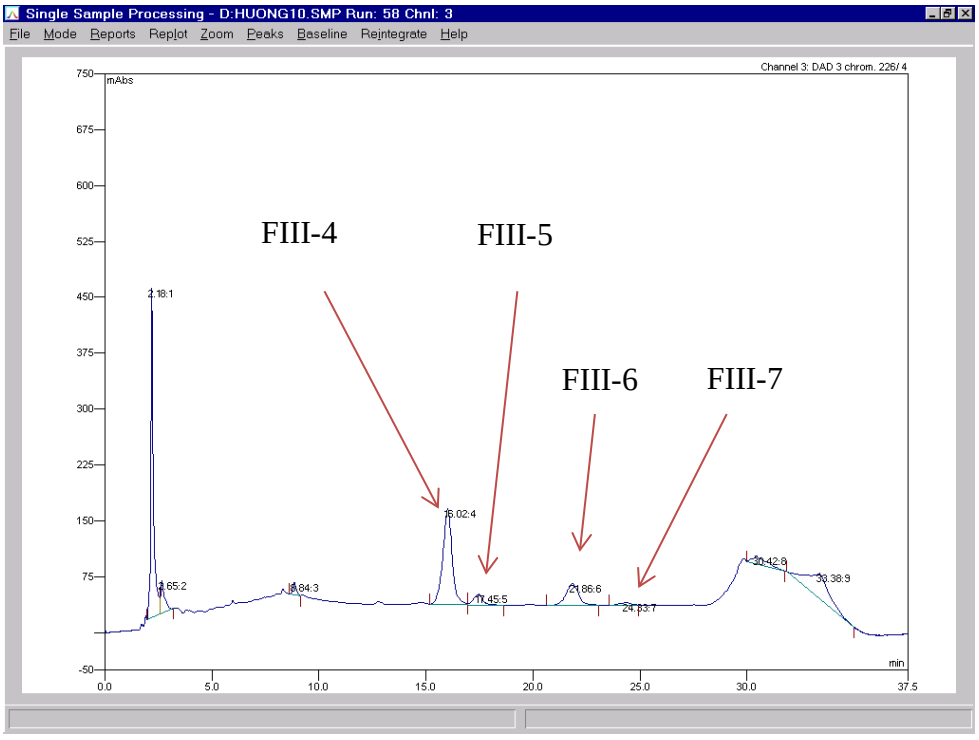


Figure III-13. HPLC of the fraction SPE 80% MeOH.

HPLC conditions: KONTRON Instruments. Column LiChrospher RP-18e (100 Å, 250x4 mm, 5 μm). Mobile phase: MeOH/H_2O 0.05% TFA. Flow rate: 1.0 mL min^{-1}. Detection wavelength: 226 nm. Running program 3. Injection: 10 μg/20 μL.

The HPLC conditions were also modified to isolate the four peaks FIII-4, 5, 6, and 7 from the SPE 80% MeOH fraction (Figure III-13). A LiChrospher RP-18e column (100 Å, 250x4 mm, 5 μm) with a step gradient of methanol in water plus 0.05% TFA as mobile phase was used with a flow rate of 1 mL min^{-1} (program 3, see II.4.6.3). For an effective separation of peaks, the amount of SPE 80% MeOH fraction should not exceed 200 μg/injection.

Further purification of FIII-4 using a LiChrospher RP-18e column (100 Å, 250x4 mm, 5 μm) with mobile phase MeOH/H_2O plus 0.05% TFA, a flow rate of 1.0 mL min^{-1} and HPLC program 4 (see II.4.6.2) yielded the major component named compound 1 (Figure III-14a). The structure of 1 was first elucidated using a combination of high-resolution ESI MS and 2D NMR techniques.

a) Compound 1 (FIII-4) b) Compound 2 (FIII-5)

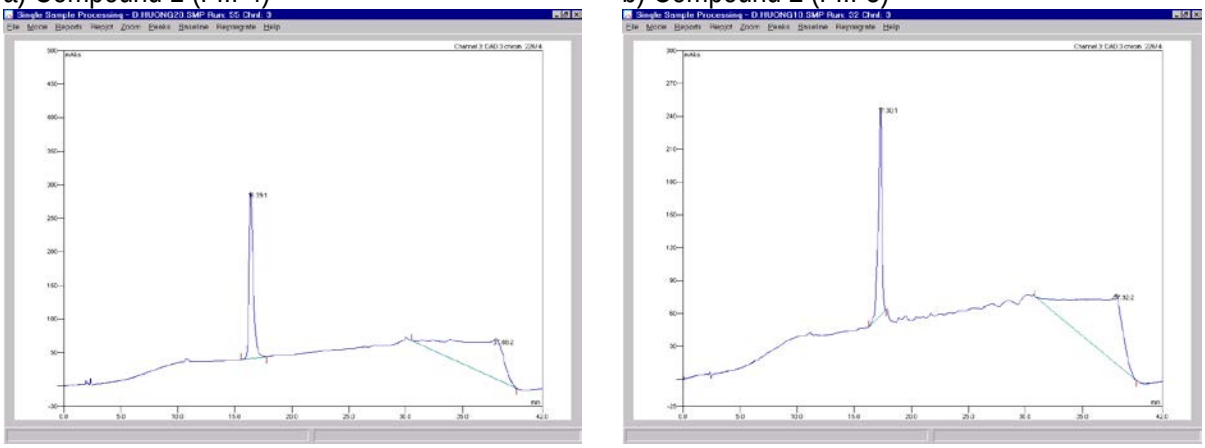


Figure III-14. Analytical HPLC chromatogram of compound 1 and 2.

a) Compound 1 (FIII-4, HPLC program 2, t_R= 16.39 minute), b) Compound 2 (FIII-5, HPLC program 2, t_R= 17.30 minute). HPLC conditions: KONTRON Instruments. Column LiChrospher RP-18e (100 Å, 250x4 mm, 5 μm). Mobile phase MeOH/H_2O 0.05% TFA. Flow rate 1.0 mL min^{-1}. Detection wave length 226 nm.

High resolution ESI mass spectrometry of fractions FIII-4, FIII-5, FIII-6 and FIII-7 (Table III-5) yielded the exact mass of the components in each fraction. Peaks FIII-6 and FIII-7 was further purified using HPLC program 6 yield compound 3 and compound 4 (Figure III-15).

a) Compound 3 (FIII-6) b) Compound 4 (FIII-7)

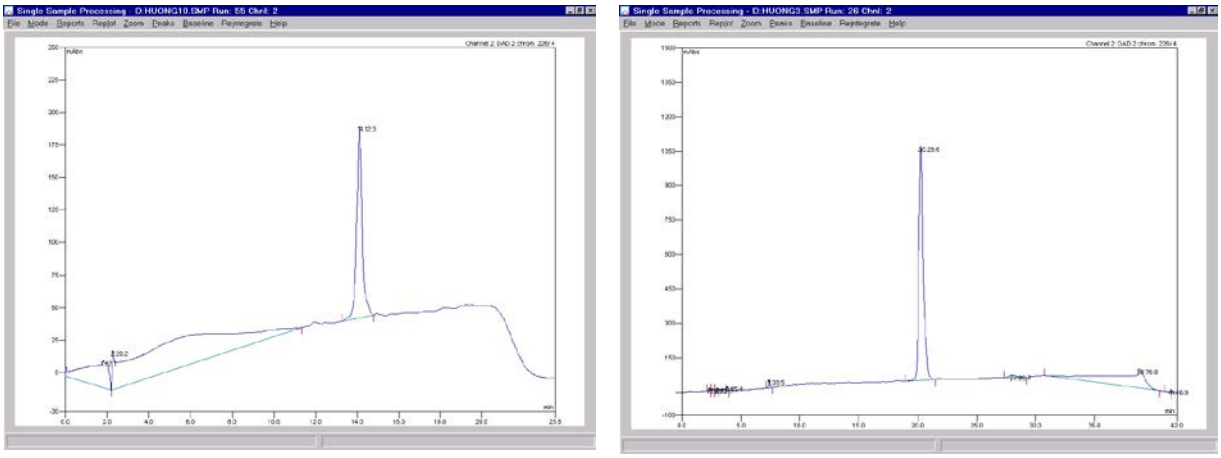


Figure III-15. Analytical HPLC chromatogram of compound 3 and 4.

c) Compound 3 (FIII-6, HPLC program 4, t_R = 14.12 minute). d) Compound 4 (FIII-7, HPLC program 2, t_R = 20.26 minute). HPLC conditions: KONTRON Instruments. Column LiChrospher RP-18e (100 Å, 250x4 mm, 5 μm). Mobile phase MeOH/H_2O 0.05% TFA. Flow rate 1.0 mL min^{-1}. Detection wave length 226 nm.

In parallel, the pure compound 1 was also isolated from FIII-4 with the mobile phase ACN plus 0.05% HCOOH in H_2O plus 0.05% HCOOH, flow rate 1 mL min^{-1} (FIII-4-3, HPLC program 5, t_R = 16.48 min, see II.4.6.4).

Table III-5. Identification of compounds in the HLPC chromatograms

HPLC Peak	Compound	Molecular Formula	$[M+H]^+$	$[M+2H]^+/2$	$[M+Na]^+$	$[M+2Na]^+/2$
FIII-4	1	$C_{75}H_{120}O_{36}N_{11}Cl$	1786.764	893.885		
FIII-5	2	$C_{75}H_{118}O_{35}N_{11}Cl$	1768.753	884.883	1790.736	906.863
FIII-6	3	$C_{75}H_{121}O_{36}N_{11}$	1752.809	876.906	1774.784	898.887
FIII-7	4	$C_{75}H_{119}O_{35}N_{11}$	1734.798	867.903		
FIII-4-3	1	$C_{75}H_{120}O_{36}N_{11}Cl$	1786.764	893.888		915.868

The yield of the isolation of the pure compounds from the biomass is shown in Table III-6.

Table III-6. Yield of fractions and pure compounds from isolation procedure in percentage of biomass

Pure compound	% biomass
MeOH:H_2O extract	30,30
FIII fraction	8,09
SPE80%	0,51
Compound 1	0,0306
Compound 2	0,0096
Compound 3	0,0287
Compound 4	0,0077

III.2.9. Structure elucidation of pure compounds isolated from strain Bio 33

The pure compounds were characterized by high-field NMR spectroscopy and high resolution electrospray ionization mass spectrometry (HR-ESI-MS). The HR-ESI-MS afforded a range of the molecular formulae compatible with the exact mass of each component (Table III-5).

Studies in various solvent mixtures indicated the best resolved 1D ^1H NMR spectra were produced in a 1:1 mixture of trifluoroethanol-d_2 and water (Index_Figure 1S1). This solvent was then used for the

extensive NMR investigation of the three components (1-3) that were available in sufficient amounts to allow structural elucidation by high-field NMR spectroscopy.

The combination of 2D ^{1}H NMR techniques and MS data of 1 allowed the identification of a linear peptide. The positive HR-ESI-MS of 1 showed the $[M+H]^+$ ion at *m/z* 1786.764 which is compatible with a molecular formula of $C_{75}H_{120}O_{36}N_{11}Cl$ (calcd. for $[M+H]^+$ 1786.766, see Table III-5).

Peptide moiety: The NMR spectra indicated the peptide moiety was the same in each of the three components and the approach used was essentially the same and is illustrated here by analysis of 1. Initially spin systems in the homonuclear ^{1}H-^{1}H COSY and TOCSY spectra (Index_Figure 1S2 and 1S3, respectively) were identified starting from the signals of the backbone amide protons in the region 9.2 to 6.4 ppm. The signals of eight amino acid residues were found (Index_Figure 1S4). From the characteristic chemical shifts, seven of which could be identified namely Thr (x3), Dhb, Glx (x2) and Gly (Table III-9, residues 1, 2, 3, 5, 6, 7 and 9). One further residue (4 in Table III-9) possessed an AA'BB' spin system that was part of an amino acid system with NH at 8.12 and additional signals at 5.14 and 4.71 ppm. This residue was later shown to be *β*-hydroxytyrosine. These spin systems were confirmed from the H*α* and high field region of the TOCSY spectrum. One further amino acid spin system (residue 8) lacking an amide proton was then identified as ThrNMe from the correlations of its H_α and H_β at 4.94 and 4.45 ppm, respectively.

Table III-7. Sequence and structural information of the cyclic peptide moiety deduced from the NOE's found in the 2D NOESY spectrum of 1

α-N (*i*, *i*+1)	N-N (*i*, *i* +1)	Others
1-2	1-2	
2-3	2-3	
3-4	3-4	
4-5	4-5	4H2/6-5NH
6-7[a]	5-6	5H_β-6NH
8-9	6-7	8NMe-8H_α
10-1[b]		8NMe-8H_β
		8H_α-9H_α

Spectra are found in the Index as detailed in the text. [a]Not observed in the series of spectra due to irradiation of the H_α under the water signal. It was observed under different conditions in other spectra. [b]There are also correlations of 10ß with 1NH.

Sequence specific assignments were determined from the cross-peaks in the 2D ^{1}H NOESY spectrum (Index_Figure 1S6) based on short observable distances between H_α and HN of amino acid *i* and HN of amino acid *i*+1 (Index_Figure 1S7). Confirmatory evidence was provided by the NH-NH correlations in

the low field region (Index_Figure 1S8). The full assignments and chemical shift data are presented in Table III-9. The data in Table III-7 afforded an unambiguous sequence of the nine residues Thr-Thr-Thr-HOTyr-Dhb-Gln-Gly-ThrNMe-Gln. The NMe substituent attached to the nitrogen of residue 8 was identified from intra-residue NOEs of the methyl group at 3.08 ppm with those of the H_α and H_β of the same residue. Confirmatory sequence correlations exist between 5NH and H2/6 of residue 4 (HOTyr) and 6NH and H_β of residue 5 (Dhb). The strong intra-residue NOE between H_γ (Me) and NH of the Dhb defines the stereochemistry of the double bond of residue 5.

Although there was insufficient material to record directly 1D ^{13}C NMR spectra, the availability of a high-field 600 MHz NMR instrument with a cryo-probehead allowed the measurement of 2D ^{1}H-^{13}C HSQC, 2D-edited HSQC, HSQC-TOCSY and HMBC spectra. Hence, ^{13}C chemical shifts of the protonated carbons of 1 were established directly from the 2D HSQC spectrum (Index_Figure 1S9). These data confirmed the nature of the amino acid systems present. Strong correlations in the HMBC spectra (Index_Figure 1S10) from the methyl groups confirmed the presence of four Thr residues. The nature of residue 4 was also confirmed by the same HMBC spectra. The shifts of all the aromatic carbons followed from the internal correlations with the aromatic protons. The proton (H_β) at 5.18 ppm showed a direct correlation with the carbon (C_β) at 74.8 ppm and long-range correlations with an aliphatic carbon (C_α) at 62.6 ppm and aromatic carbons C1 and C2/6. In addition, the reverse correlation of H2/6 with the carbon at 74.8 ppm was also evident, thus confirming residue 4 is a β-hydroxytyrosine unit.

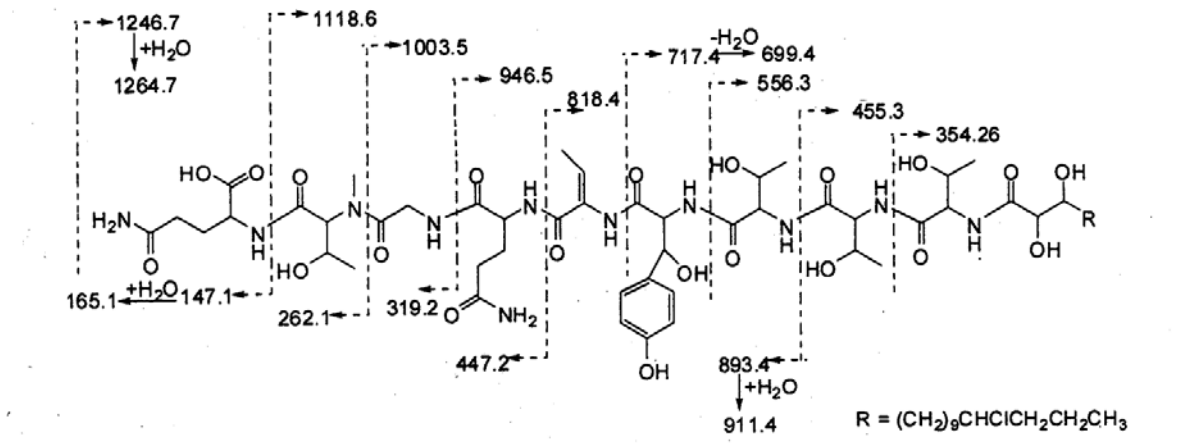


Figure III-16. MS^n fragmentation ions from the ESI-MS of 1 that confirm the peptide sequence.

The quality of the HMBC spectrum was sufficient to establish the assignment of the carbonyl groups. Hence, correlations from the H_α and H_β protons allowed direct assignment of the intra-residue carbonyl carbons while correlations of the NH protons afforded the assignment of the carbonyl carbons of the

preceding residue in the sequence. These data again yielded independent sequence information that was compatible with the result from the NOE data. Finally the amino acid sequence of 1 was confirmed from a detailed analysis of the b and y-ions of a high resolution ESI-MS data of the peptide fragments from the MS^2 of the $[M+2H]^{2+}$ ions in Table III-10, Index_Figure 1S11, and Figure III-16.

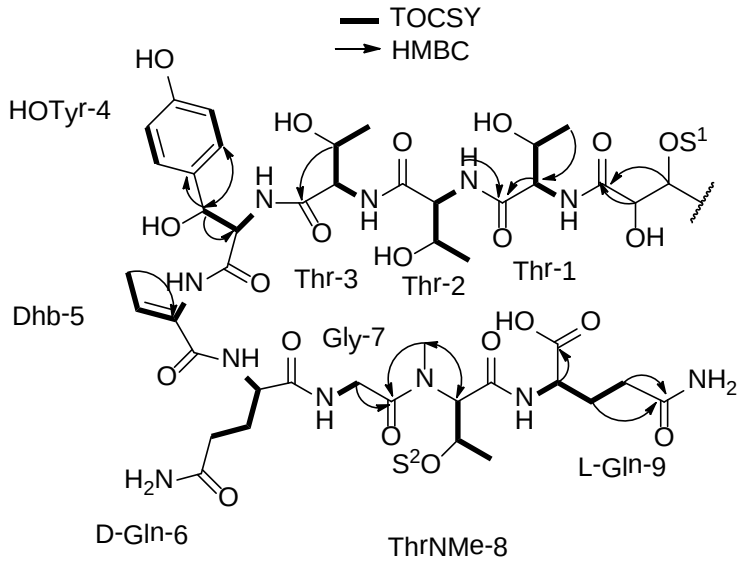


Figure III-17. Selected ^1H-^1H COSY, TOCSY and HMBC correlations of 1.

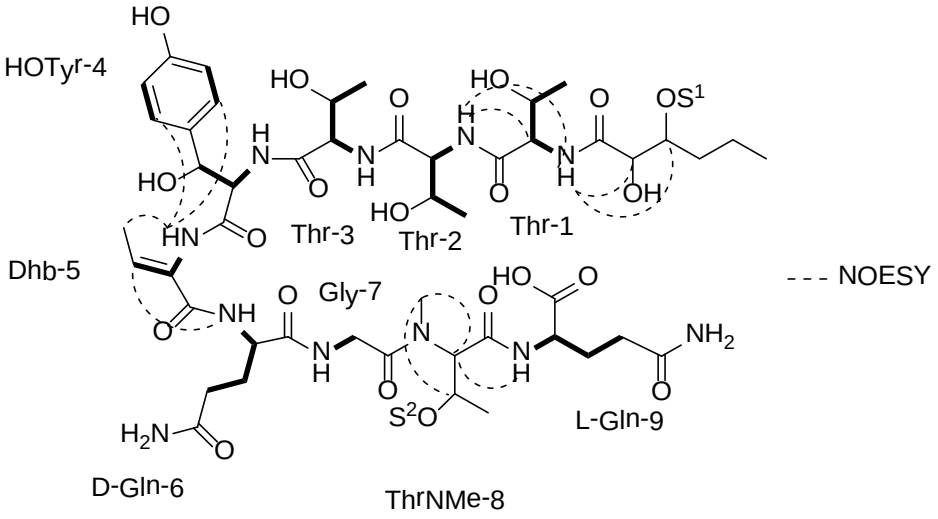


Figure III-18. Selected NOESY correlations of 1.

A literature search using partial sequences revealed a closely related cyclic peptide, hassallidin A (see page 19) (Neuhof *et al.*, 2005; Dieckmann *et al.*, 2006) in which the same sequence was present apart from the replacement of the 4OHTyr unit by Tyr. In this system ring closure occurred through the formation of a lactone bridge between the hydroxyl group of the second Thr system in the sequence and the carboxylic acid of the terminal Gln. This was identified by the downfield acylation shift of ca. 0.8 ppm

in the ^{1}H NMR spectrum of H_{β} and ca. 4-5 ppm upfield shift of C_{γ} of the substituted Thr compared to the other threonines in the molecule. This was not the case in our data of 1 and 3 but was compatible with the data of 2. Thus the low field shift of the H_{β} of 2-Thr in 2 indicated that ring closure had taken place at this position as in hassallidin A. The IR spectrum of 2 showed absorption at 1737 cm^{-1} close to the absorption given for hassalidin A (1740 cm^{-1}) and typical for the presence of an ester/lactone structure which was absent in 1 and 3.

Amino acid analysis confirmed the presence of Gly, Glx, Thr, ThrNMe and *allo*-Thr. Variable temperature-time analysis of the enantiomeric labelled total hydrolysate of 2 by chiral gas chromatography – mass spectrometry (GC-MS) indicate the presence of variable amounts of the D and L enantiomers of Glu (=Gln). Under mild conditions (80°C for 2 h) only the L-enantiomer of Glu (=Gln) was present, while under more stringent conditions (100°C: 3 h) both D- and L-enantiometers in a ratio of 1:1 were found. A similar ratio was found when 1 was hydrolysed at 120°C for 12 h. As one Gln is a terminal residue, and according to MS/MS experiments readily cleaved, this residue must be the L-enantiometer. As the D and L composition remains constant under the more extreme conditions, clearly racemization is not occurring. Hence the "inner" Gln must have the D configuration. This hypothesis that no racemization occurs is further justified as only one enantiomer of ThrNMe appeared under relatively mild conditions and relatively easily cleaved in MS/MS. The chirality of ThrNMe is unclear as reference material was not available.

The data for Thr is more complex. Independent of hydrolysis conditions both D and L-enantiomeric forms of Thr were detected in a ratio of approximately 2:1, may be one Thr comes from Dhb, because racemization seems to be improbable. In addition, a further single peak of the same intensity as the L-Thr peak was present and showed a similar fragmentation pattern to those of Thr. This peak was identified as D-*allo*-Thr. From our point of view the position of the different enantiomers of Thr in the amino acid sequence can only be clarified by X-ray crystallography, but crystallization of the molecule was not successful. The absence of reference material prevented the analysis of the other amino acid residues present. In general, our data are compatible with those observed for hassallidin A, but we have now elucidated the position of the D and L-forms of Gln in the peptides.

Acyl side chain: The 1D and 2D NMR spectra of each component showed a considerable number of spin systems that did not belong to the proteinogenic amino acids. A 2,3-dihydroxy aliphatic acid derivative (10DhA) with signals in both the region 5.5 to 3.0 ppm (Table III-9) and high field aliphatic region was present. This is an acyl residue attached to the amidic nitrogen of residue 1-Thr from the NOESY correlation of its H_{α} and H_{β} at 4.28 and 4.07 ppm, respectively, with NH of 1-Thr at 8.03 ppm (Index_Figure 1S7). The exact nature of this system was initially difficult to unambiguously identify from

the NMR data alone although this system showed the same signals for the aliphatic chain-end in 1 and 2, but were different in 3. In contrast, the carboxyl terminal region was identical in all three compounds. The 2D HMBC spectrum of 1 showed correlations from the terminal methyl group that identified the ^{1}H and ^{13}C chemical shifts of the CH_3 CH_2 CH_2 chain end with ^{13}C shifts of 14.1, 20.9, and 41.9 ppm, respectively (see Index_Figure 1S10) and indicate the second methylene group is adjacent to a carbon carrying a heteroatom which is absent in 3. A detailed comparison of the HRESI/MS/MS of $[M+2H]^+$ for 1 and 3 (Table III-10) indicated the presence of a chlorine versus hydrogen exchange in a side chain attached to a terminal Thr unit as the only difference between these two molecules. The same data allowed the molecular formula and the exact chain length of this moiety in 1, a derivative of 2,3-dihydroxy-13-chlorohexadecanoic acid (palmitic acid), and those of compounds 1-3 to be verified.

Sugar units: In the ^{1}H NMR spectra of all three compounds there were a number of systems in the region 5.2 to 3.3 ppm, with no corresponding high-field aliphatic signals, which clearly belonged to sugar-like residues (Index_Figure 1S5a, b, c). This was in agreement with a sugar compositional analysis which indicated the presence of mannose, arabinose and galacturonic acid. Similar sets of signals were found in the NMR spectra of each compound indicating these units were present in all the elucidated compounds. In each case three systems (S1, S2, S3) in which the lowest field signals (anomeric protons at 5.12, 5.03 and 4.60 ppm, respectively) correlated with characteristic anomeric carbons in the region 96-103 ppm (Index_Figure 1S9). The lowest field anomeric proton (S1, 5.12 ppm) was part of a five-membered spin proton system (Index_Figure 1S5a) and showed strong NOE signals to the γCH_2 group of the 10DhA unit indicating this was substituted at C_β of this unit. The magnitude of the coupling constants, particularly the presence of a geminal pair, indicated this belonged to a β-arabinose system (Table III-9). The second sugar unit, S2, with an anomeric proton at 5.03 ppm (Index_Figure 1S5b) was attached at C_β of 8-ThrNMe from its correlation in the NOESY spectrum with the signal at 4.45 ppm (H_β of 8-ThrNMe). The sugar analysis and NMR data (Index_Figure 1S5b, Table III-9) indicated this unit belonged to a mannose system.

A third unit in 1 showed an anomeric proton at 4.60 ppm with a vicinal coupling of 7.7 Hz characteristic of a chair conformation of a β-pyranose system and a readily identified spin system in the TOCSY spectra consisting of five protons (Index_Figure 1S5c). This was only compatible with the galacturonic acid found in the sugar analysis. The position of this unit at C3 of arabinose follows from the observation in 3 of NOE's from the anomeric proton (4.62 ppm) to H3 and H4 of S1 (4.25 and 4.05, respectively) in the NOESY spectrum of 3 and of its correlation with C3 of arabinose in the HMBC spectrum (Index_Figure 3S9).

In each compound the HRESI/MS/MS showed the $[M+2H]^{2+}$ ion underwent readily sequential loss of a hexose, pentose and hexuronic acid units and was compatible with the sugar compositional analysis which indicated these units corresponded to mannose, arabinose and galacturonic acid. Enantioselective carbohydrate analysis revealed the enantiomerity of the monosaccharide residues as D(+)-mannose, D(-)-arabinose and D(+)-galacturonic acid.

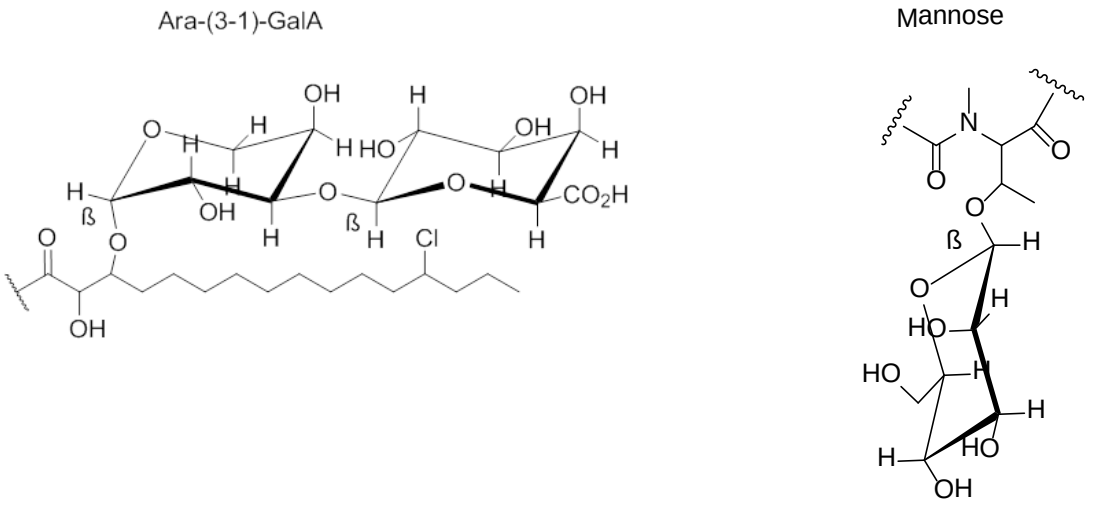


Figure III-19. Sugar units of 1

The peptide sequence of 1 - 4 was confirmed from fragmentation pattern in HR-ESI-MS. The same procedure indicated the identity and sequence of the peptide unit was the same in all four compounds. Because of insufficient amounts of 4 no NMR data are available and the structure of 4 was deduced from fragmentation pattern and *m/z* 1734.798 $[M+H]^+$ compatible with the molecular formula of $C_{75}H_{120}O_{35}N_{11}$ and the calculated mass of 1734.794. Furthermore, the IR spectrum of 4 showed absorption at 1732 cm^{-1} in the range typical for esters (lactone) and similar to the absorption of hassalidin A (1740 cm^{-1}) and balticidin B (1737 cm^{-1}), so that a cyclic structure is supposed.

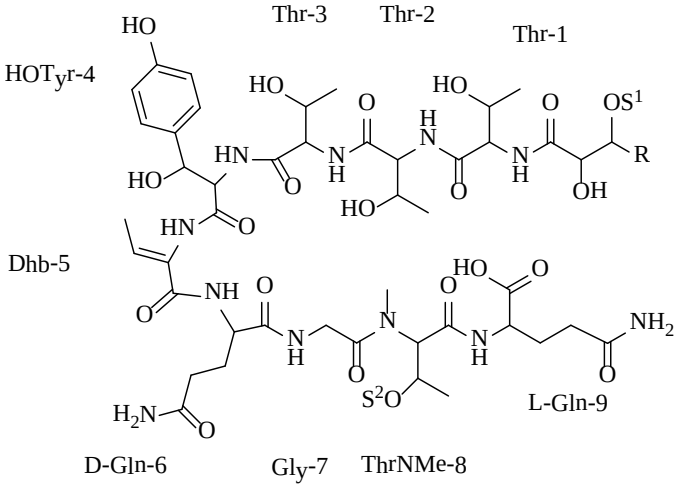
From all HR-ESI-MS and NMR data the following structures of compounds 1 – 4 were deduced and the substances named as balticidins 1 – 4. The peptide sequence (1-9) is: R.CHO(S^1).CHOH.CONH-Thr(1)-Thr(2)-Thr(3)-HOTyr(4)- Dhb(5)-Gln(6)-Gly(7)-ThrNMe(8)(S^2)-GlnCO_2H(9)

Balticidin A (1): white, amorphous powder, UV (CH_3OH/H_2O) λ_{max} 223 and 272 nm; IR ν_{max} (film) 3191, 2939, 2841, , 1662, 1411, 1190, 1140, 1085, 1027, 968, 922, 882, 799, 722, 674, 640, 546, 471 cm^{-1};^{1}H and ^{13}C NMR in CF_3CD_2OH/H_2O (1:1), see Table III-9; MS-MS fragmentation, see Table III-10; HRESIMS *m/z* 1786.764 $[M+H]^+$, *m/z* 893.885 $[M+2H]^{2+}$ (calcd for $C_{75}H_{121}O_{36}N_{11}Cl$, 1786.766).

Balticidin B (2): white, amorphous powder, UV (CH_3OH/H_2O) λ_{max} 226 and 274 nm; IR ν_{max} (film) 3382, 3262, 3201, 2929, 2365, 1737, 1660, 1628, 1616, 1563, 1537, 1512, 1456, 1431, 1405, 1241, 1202,

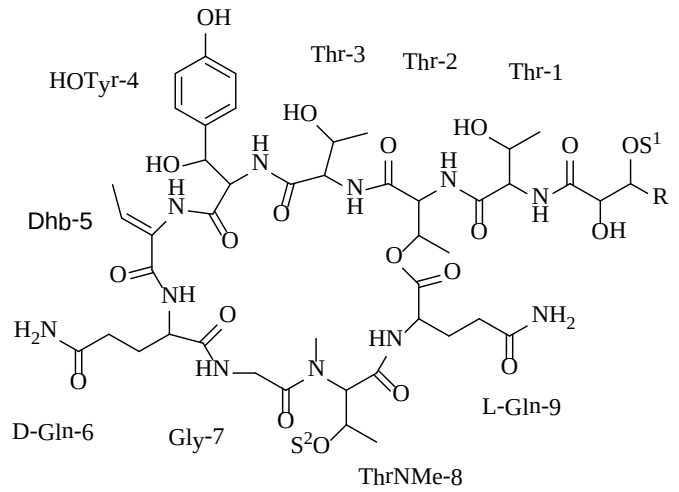
1135, 1077, 1008, 973, 916, 820, 802, 773, 669, 645, 590, 520 cm^{-1}; ^{1}H and ^{13}C NMR in CF_3CD_2OH/H_2O (1:1), see Table III-9; HRESIMS *m/z* 1768.753 $[M+H]^+$, *m/z* 884.883 $[M+2H]^{2+}$ (calcd for $C_{75}H_{119}O_{35}N_{11}Cl$, 1768.755).

A and **C**



A (1): S^1 = Ara(3-1)GalA S^2 = Man R = $(CH_2)_9CHClCH_2CH_2CH_3$
C (3): S^1 = Ara(3-1)GalA S^2 = Man R = $(CH_2)_9CH_2CH_2CH_2CH_3$

B and **D**



B (2): S^1 = Ara(3-1)GalA S^2 = Man R = $(CH_2)_9CHClCH_2CH_2CH_3$
D (4): S^1 = Ara(3-1)GalA S^2 = Man R = $(CH_2)_9CH_2CH_2CH_2CH_3$

Figure III-20. Structure of balticidins A – D.

Balticidin C (3): white, amorphous powder, UV (CH_3OH/H_2O) λ_{max} 227 and 273 nm; IR ν_{max} (film) 3389, 3309, 2926, 2841, ,1654, 1612, 1518, 1454, 1407, 1203, 1135, 1073, 975, 922, 839, 800, 725, 669 cm^{-1}; ^1H and ^{13}C NMR in CF_3CD_2OH/H_2O (1:1), see Table III-9; MS-MS fragmentation, see Table III-10; HRESIMS *m/z* 1752.809 $[M+H]^+$, *m/z* 876.906 $[M+2H]^{2+}$ (calcd for $C_{75}H_{122}O_{36}N_{11}$, 1752.805).

Balticidin D (4): white, amorphous powder, UV (CH_3OH/H_2O) λ_{max} 224 and 273 nm; IR ν_{max} (film) 3465, 3358, 3262, 2924, 2853, 1732, 1656, 1532, 1422, 1384, 1261, 1238, 1201, 1136, 1065, 1033, 1006, 975, 912, 840, 801, 721 cm^{-1}; HRESIMS *m/z* 1734.798 $[M+H]^+$, *m/z* $[M+2H]^{2+}$ 867.903 (calcd for $C_{75}H_{120}O_{35}N_{11}$, 1734.794).

Table III-8. Molecular weight calculation for 1 as a linear molecule without any ring closure

Residue	Molecular formula of fragment	Observations
1-Thr	$C_4H_7NO_2$	
2-Thr[a]	$C_4H_7NO_2$	No ring closure here
3-Thr	$C_4H_7NO_2$	
4-HOTyr	$C_9H_9NO_3$	
5-DeThr	C_4H_5NO	
6-Gln	$C_5H_8N_2O_2$	
7-Gly	C_2H_3NO	
8-ThrNMe	$C_5H_8NO_2$	
9-Gln	$C_5H_9N_2O_3$	Open chain
Fragment total	$C_{42}H_{63}N_{11}O_{18}$	
Man	$C_6H_{11}O_5$	On ThrNMe
Ara	$C_5H_8O_4$	Bound to side chain at C2
GalA	$C_6H_9O_6$	Bound to Ara
Fragment total[b]	$C_{17}H_{28}O_{15}$	
10Dht	$C_{16}H_{29}O_3Cl$	Calculated from smallest fragment containing chlorine atom ($[M+H]^+$ 388.223)
M	$C_{75}H_{120}N_{11}O_{36}Cl$	
$[M+H]^+$	$C_{75}H_{121}N_{11}O_{36}Cl$ Calc. 1786.766 Found 1786.764	

Relationship between 1- 4 is as follows from the molecular mass differences:
1 to 2 is 18.008 mass units (loss of H_2O resulting from ring closure at Cß of 2-Thr)
3 to 4 is 18.010 mass units
1 to 3 is 33.964 mass units (exchange of Cl for H in C-16 side-chain)
2 to 4 is 33.966 mass units

Table III-9. ^{1}H and ^{13}C NMR data of 1-3 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

Residue	C/H No.	δ_C 1	δ_H 1	δ_C 2	δ_H 2	δ_C 3	δ_H 3
1-Thr	NH		8.03		8.03		8.03
	CO[c]	173.7		169.0		173.9	
	C_α	62.1	4.34	65.0	4.01	61.9	4.34
	C_β	69.4	4.17	67.7	4.01	69.5	4.16
	C_γ	21.0	1.30	23.0	1.42	20.9	1.30
2-Thr	NH		8.51		9.48		8.53
	CO	175.3		172.8		175.4	
	C_α	60.8[e]	4.48	57.3	4.70	61.1	4.48
	$C_{\beta(ring)}$	69.7	4.37	73.2	5.72 attached to 171.7	69.7	4.38
	C_γ	20.8	1.28	18.7	1.27	21.0	1.26
3-Thr	NH		8.42		8.55		8.42
	CO	174.1		176.7		174.1	
	C_α	62.3	4.37	62.9	4.28	62.4	4.36
	C_β	69.2	4.21	69.9	4.17	69.1	4.20
	C_γ	20.4	1.23	23.8	1.44	20.4	1.22
4-HOTyr[f]	NH		8.12		8.33		8.14
	CO	173.5[c]		nd		173.4	
	C_α	62.6	4.71	63.1	4.60	62.7	4.71
	C_β	74.8	5.18	73.1	5.39	74.7	5.12
	Arom	C1:133.0, C2,6:130.2 C3,5:117.8 C4: 158.3	H2/6:7.37 H3/5:6.94	134.0 129.8 117.6 158.1	7.40 6.96	132.9 130.2 117.7 158.3	7.36 6.93
5-Dhb	NH		9.09		9.37		9.11
	CO	168.0[c]		166.1		168.0	
	C_α	129.4		nd		129.5	
	C_β	138.0	6.78	141.1	6.78 (7.10)	138.1	6.77
	C_γ	14.1	1.48	14.6	1.71	14.1	1.46
6-Gln	NH		7.88		7.36		7.88
	CO	175.3[c]		175.2		175.5	
	C_α	55.3	4.54	53.3	4.87	55.3	4.53
	C_β	29.4	2.26, 2.07	32.7	2.07, 2.29	29.4	2.26, 2.06
	C_γ	33.2	2.42	32.7	2.39, 2.17	33.1	2.41
	$CONH_2$	180.1[c]	7.43, 6.58[a]	nd	7.60, 6.57	180.1	7.43, 6.66
7-Gly	NH		8.13		8.65		8.16
	CO	172.9[c]		174.8		172.8	
	C_α	43.4	4.20, 4.13	43.7	4.31, 3.87	43.4	4.18, 4.12
8-ThrNMe	NMe	32.8	3.08	32.2	3.03	32.8	3.07
	CO	171.5[c]		169.4		171.5	
	C_α	62.9	4.94	62.5	5.06	62.7	4.95
	C_β	70.7	4.45	69.9	4.47	70.7	4.43
	C_γ	15.9	1.19	15.3	1.17	15.9	1.18
9-Gln	NH		7.85		8.74		8.02
	CO	nd		nd		nd	
	C_α	56.9	4.27	54.2	4.70	56.0	4.34
	C_β	29.8	2.14, 1.97	31.5	1.87, 1.68	29.3	2.17, 1.99
	C_γ	33.7	2.29	33.8	2.24, 2.11	33.4	2.31
	$CONH_2$	180.0	7.42, 6.64[a]	nd	7.48, 6.30	180.0	7.43, 6.62
10-DhA	CO	177.1[c]		177.1		177.2	
	CαHO	74.6	4.28	74.6	4.27	74.6	4.27
	CβHO	76.4	4.07[b]	75.5	4.14	76.4	4.07
	CH_2	29.3	1.70-1.75	28.0	1.80, 1.67	29.3	1.71
	CH_2	27.3	1.33-1.39	27.7	1.39	27.3	1.35
	$(CH_2)_n$	30.8	1.31	30.8	1.31	30.9	~1.3
	CH_2	39.9	1.69	39.9	1.71		
	CHCl	66.8	3.99	66.9	3.99		
		41.9	1.69	41.9	1.71	33.3	1.27

		20.9	1.52,1.42	21.5	1.53, 1.43	24.0	1.29
	CH_3	14.1	0.91	14.1	0.92	14.9	0.87
S1: ß-arabinose Attached to CβHO of 10-DhA	C1	96.9	5.12(bs, J_{12} small)	95.6	5.15 (dd, J_{12} 3.9)	96.8	5.13(bs, J_{12} small)
	C2	68.9	3.95	68.8	3.99(dd, J_{23} 10.2)	68.8	3.97
	C3	77.4	4.26	76.9	4.40(bd, J_{34} small)	77.5	4.25
	C4	68.9	4.05	69.0	4.23	68.9	4.04
	C5	64.7	3.43 (bd, J_{45A} small), 3.34 (bd, J_{45A} small, J_{5A5B} 11.7)	64.7	3.55 (bd, J_{45A} small), 3.42 (bd, J_{45B} small, J_{5A5B} 11.6)	64.7 (t)	3.49 (bd, J_{45A} small), 3.38 (bd, J_{45A} small, J_{5A5B} 11.9)
S2: Mannose unit Attached to 8-ThrNMe	C1	98.4	5.03[d]	98.7	5.02(bs, J_{12} small)	98.4, 98.4	5.04
	C2	73.1	3.93	73.3	3.81(bs, J_{23} small)	72.9 x2	3.93
	C3	72.8	3.84	72.8	3.48(bd,J_{34} 9.5)	72.8	3.84
	C4	69.1	3.70	68.5	3.75(dd, J_{45} 9.7)	68.9	3.75
	C5	75.0	3.61	74.7	3.54	75.0	3.61
	C6	nd	3.93	62.8	3.85	62.9 (t)	3.93
S3: Galacturonic acid unit	C1	102.3	4.61(d, J_{12} 7.7)	101.3	4.70(d, J_{12} 7.8)	102.0	4.62
	C2	72.8	3.68	(72.8)	3.69	72.7	3.69
	C3	75.3	3.75	(75.5)	3.72	75.1	3.76
	C4	72.8	4.29	72.8	4.29[e]	72.5	4.30[e]
	C5	nd	4.04	nd	4.12	77.4	4.12
	C6	nd	-	nd	-	nd	-

[a]Interchangeable. [b]Unambiguous assignment from COSY data. [c]Unambiguous assignment from HMBC. [d]Mannose unit assigned by comparison with **3**. [e]The intensities of the cross peak in the TOCSY spectrum indicates J_{45} is small. [f]Hydroxyl groups are found attached to the ß carbon atom and C4 of the aromatic system. nd Not detected..

Table III-10. Comparison of high resolution ESI MS data of the peptide fragments of 3 and 1 from the MS^2 of the $[M+2H]^{2+}$ ions at *m/z* 876.908 and 893.885, respectively[a].

3					1			
Fragment	Measured	Calculated	Formula	Assignment	Measured	Calculated	Formula	Assignment
a	147.076	147.076	$C_5H_{11}O_3N_2$	Gln+H2O	147.076	147.076	C5H11O3N2	
b	262.140	262.140	$C_{10}H_{20}O_5N_3$	Gln+N-Me-Thr	262.140	262.140	C10H20O5N3	
c	319.161	319.161	$C_{12}H_{23}O_6N_4$	b + Gly	319.161	319.161	C12H23O6N4	
d[b]	354.263	354.264	$C_{20}H_{36}O_4N$		388.223	388.225	C20H35O4NCl	d +Cl-H
e	447.219	447.220	$C_{17}H_{31}O_8N_6$	c +Gln	447.220	447.220	C17H31O8N6	
f	455.311	455.312	$C_{24}H_{43}O_6N_2$	d + Thr	489.272	489.273	C24H42O6N2Cl	f +Cl-H
g	556.359	556.359	$C_{28}H_{50}O_8N_3$	f+Thr	590.320	590.320	C28H49O8N3Cl	g+Cl-H
h	717.407	717.407	$C_{37}H_{57}O_{10}N_4$	g+OH-Tyr-H2O	751.368	751.368	C37H56O10N4Cl	h +Cl-H
i	818.454	818.455	$C_{41}H_{64}O_{12}N_5$	h +Thr	852.416	852.416	C30H62O19N9	i +Cl-H
j	893.399	893.400	$C_{38}H_{57}O_{15}N_{10}$	o +Thr+H2O	893.400	893.400	C38H57O15N10	
k	946.512	946.513	$C_{46}H_{72}O_{14}N_7$	i +Gln	980.475	980.474	C46H71O14N7Cl	k +Cl-H
l	1003.534	1003.535	$C_{48}H_{75}O_{15}N_8$	k+Gly	1037.496	1037.496	C48H74O15N8Cl	l +Cl-H
m	1118.596	1118.598	$C_{53}H_{84}O_{17}N_9$	l+NMe-Thr	1152.559	1152.559	C53H83O17N9Cl	m+Cl-H
n	1246.653	1246.657	$C_{58}H_{92}O_{19}N_{11}$	m+Gln	1280.618	1280.618	C58H91O19N11Cl	n +Cl-H
o	1264.664	1264.667	$C_{58}H_{94}O_{20}N_{11}$	n +H2O	1298.627	1298.628	C58H93O20N11Cl	o+Cl-H

Footnotes: [a] The peptide sequence follows from the N-terminal fragments (d-f-g-h-i-k-l-m-n) and carboxy-terminal fragments (a-b-c-e-j). [b]Fragments d of **3** and **1** were found in the MS^3 of ions at *m/z* 717 and 751, respectively.

III.2.10. Antimicrobial activity of pure compounds from strain Bio 33

The activity against *Candida maltosa* of the four isolated balticidins was confirmed in the agar diffusion test. The inhibition zones were 12, 15, 9 and 18 mm for compounds 1, 2, 3 and 4, respectively (the amount of test sample was less than 10 μg). These compounds also showed no inhibition zone against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*. These results confirmed the specific antifungal activity of those compounds.

III.3. Growth and antifungal activity of strain Bio 33 under different cultivating conditions

NaCl was added to all media at concentration of 5 g/L medium. The growth of strain Bio 33 cultivated in a standard condition BG 11 plus 0.5% NaCl at 22.5°C, 20 μmol photons m-2 s-1 , 12 h/12 h light/dark rhythm was used as reference. According to the reference growth curve, the period from day 0^{th} to 5^{th} is the lag phage in which the cyanobacteria adapted itself to the new culture conditions. Hence, the specific growth rate μ and the mean doubling time G were calculated from 5^{th} to 20^{th} day and from 20^{th} to 30^{th} day.

III.3.1. Effect of nutrient medium on growth of Bio 33

Strain Bio 33 was cultivated in BG 11 and MBL medium containing 0.5% NaCl (see II.1.2.1). The content of $NaNO_3$ changes from 1.5 g/L in BG 11 to 0.085 g/L in MBL. The N:P ratio is 100:1 in BG 11 medium and 20:1 in MBL medium.

From the growth curves, the time period from 0 – 5^{th} day was considered as the lag phase in which strain Bio 33 adapted itself to culture conditions. The exponential phase of strain Bio 33 was the time from 5^{th} to 20^{th} day or 5^{th} to 30^{th} day depending on culture conditions. In this period, the increasing of biomass was obviously visible because an exponential increasing growth rate correlated with reduction of generation time. Generally, the difference in growth of strain Bio 33 between BG 11 medium and MBL was obviously observed after day 20^{th}. In most cases, the biomass was still increasing until 30^{th} day. From day 20^{th} to 30^{th}, the raising generation time corresponded with reduced growth rate. It is obviously that in both media, strain Bio 33 has just reached the early stationary phase at 30^{th} day when the weight of dried biomass was still increasing but a strong raise of generation time was observed.

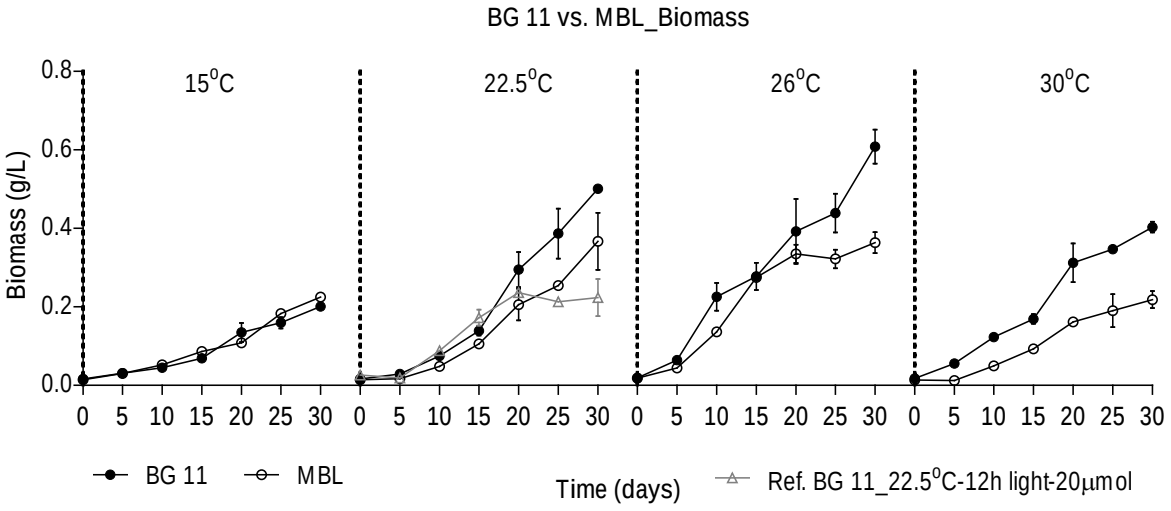


Figure III-21. Effect of nutrient medium on growth of Bio 33

NaCl was added to all media at a concentration of 5 g/L. The flasks were shaken at 75 rpm. 24 h continuous irradiance, light intensity 20 μmol photons m^{-2} s^{-1}. Bars represent the standard deviation. n = 2.

Table III-11. Specific growth rate μ and mean doubling time G of strain Bio 33 grown in BG 11 and MBL media at different temperatures (mean ± SD)

Temp. (°C)	Medium + 0.5% NaCl	μ (log_{10} unit per day)		G (days)	
		5 – 20 days	20 – 30 days	5 – 20 days	20 – 30 days
15	BG 11	0.097 ± 0.018	0.041 ± 0.020	7.3 ± 1.3	19.1 ± 9.1
	MBL	0.085 ± 0.014	0.073 ± 0.008	8.2 ± 1.3	9.5 ± 1.1
22.5	BG 11	0.155 ± 0.001	0.054 ± 0.013	4.5 ± 0.0	13.4 ± 3.3
	MBL	0.167 ± 0.032	0.058 ± 0.040	4.2 ± 0.8	15.7 ± 10.8
26	BG 11	0.120 ± 0.019	0.045 ± 0.028	5.8 ± 0.9	19.3 ± 12.2
	MBL	0.135 ± 0.006	0.008 ± 0.000	5.1 ± 0.2	85.0 ± 4.9
30	BG 11	0.115 ± 0.008	0.026 ± 0.019	6.1 ±0.4	36.5 ± 27.0
	MBL	0.170 ± 0.013	0.030 ± 0.011	4.1 ± 0.3	24.8 ± 9.3

An overview of growth curves of Bio 33 in BG 11 and MBL media at different temperatures showed that the growth of Bio 33 cultivated in BG 11 and MBL media depended on temperature. At 15°C, no considerable difference in growth of Bio 33 in BG 11 and MBL was recorded. The difference in biomass between BG 11 and MBL media was not significant ($P < 0.05$). At higher temperatures, Bio 33 grew better in BG 11 medium; the biomass produced in BG 11 medium was higher ($P < 0.05$) than in MBL medium. At 26°C - the optimum temperature for growth of Bio 33 both in BG 11 medium and

MBL, the dry weight of biomass cultivated in BG 11 medium harvested at 30th day was 1.7 fold higher than in MBL medium. The difference in biomass between these media was largest at 30°C ($P < 0.01$). In conclusion, BG 11 was the better medium for growth of Bio 33.

III.3.2. Effect of nitrate on growth of Bio 33

Bio 33 was cultivated in BG 11 medium and MBL medium with and without $NaNO_3$. NaCl was added to each medium at a concentration of 5 g/L.

III.3.2.1. *Bio 33 cultivated in BG 11 medium*

Strain Bio 33 was cultivated in BG 11 medium without $NaNO_3$ at 18, 22.5 and 26°C. The growth curves, growth rate and the doubling time are represented in Figure III-22 and Table III-12.

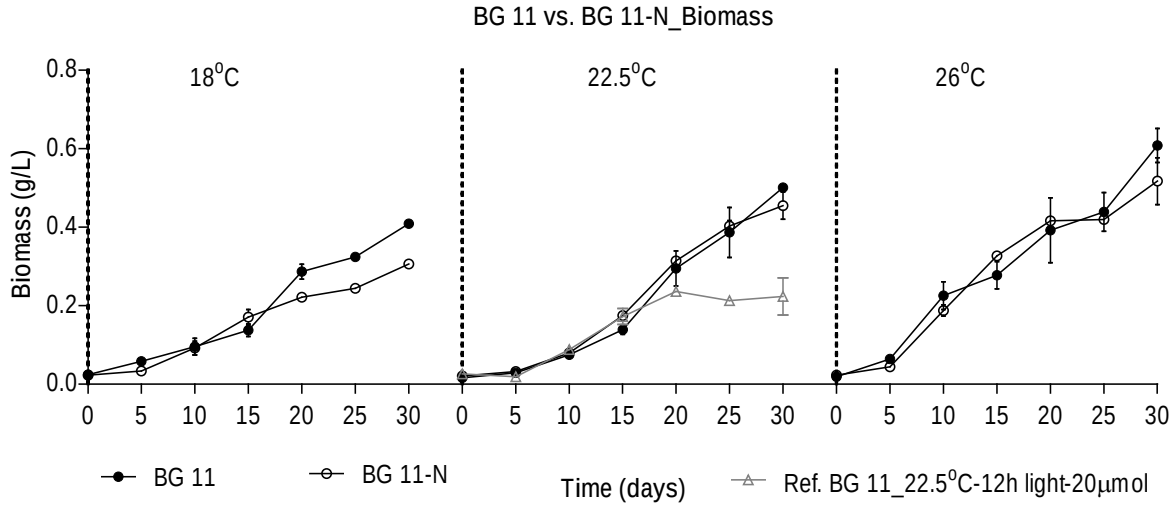


Figure III-22. Effect of nitrate on growth of Bio 33 in BG 11 medium.

Temperature: 18, 22.5 and 26°C. NaCl was added to all media at a concentration of 5 g/L. The flasks were shaken at 75 rpm. 24 h continuous irradiance, light intensity 20 μmol photons m^{-2} s^{-1}. Bars represent the standard deviation. n = 2.

It was observed that nitrate differently affected on Bio 33 at different temperatures. At 18°C, the effect of nitrate on growth of Bio 33 was remarkable after the 20th day. The difference in biomass after the 20th day was significant ($P < 0.05$). At 22.5°C and 26°C, the depletion of nitrate from BG 11 medium however did not affect the growth of Bio 33. Difference in biomass between medium with and without nitrate was not significant ($P < 0.05$).

Table III-12. The specific growth rate μ and the mean doubling time G of strain Bio 33 grown in BG 11 medium with and without $NaNO_3$ at different temperatures (mean ± SD)

Temp. (°C)	Medium + 0.5% NaCl	μ (log_{10} unit per day)		G (days)	
		5 – 20 days	20 – 30 days	5 – 20 days	20 – 30 days
18	BG 11	0.106 ± 0.002	0.036 ± 0.005	6.5 ± 0.1	19.7 ± 3.0
	BG 11 - N	0.126 ± 0.007	0.032 ± 0.001	5.5 ± 0.3	21.5 ± 0.7
22.5	BG 11	0.155 ± 0.001	0.054 ± 0.013	4.5 ± 0.0	13.4 ± 3.3
	BG 11 - N	0.152 ± 0.005	0.037 ± 0.007	4.6 ± 0.1	19.2 ± 3.7
26	BG 11	0.120 ± 0.019	0.045 ± 0.028	5.8 ± 0.9	19.3 ± 12.2
	BG 11 - N	0.150 ± 0.008	0.021 ± 0.009	4.6 ± 0.3	35.7 ± 15.5

III.3.2.2. *Bio 33 cultivated in MBL medium*

Strain Bio 33 was cultivated in MBL medium without $NaNO_3$ at 22.5 and 26°C. The growth curves, growth rate and the doubling time are represented in Figure III-23 and Table III-13.

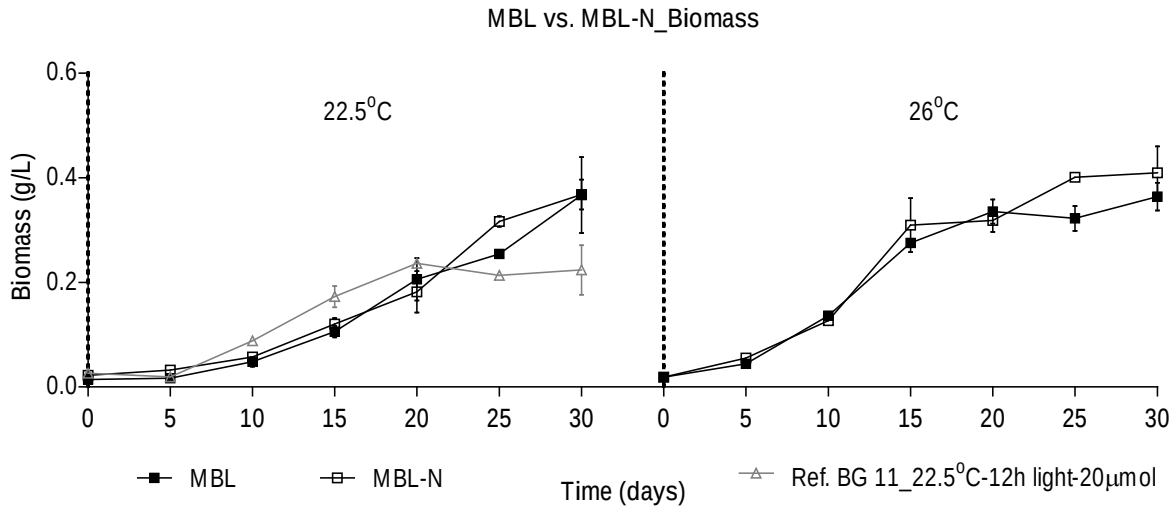


Figure III-23. Effect of nitrate on growth of Bio 33 in MBL medium.

Temperature: 22.5 and 26°C. NaCl was added to all media at a concentration of 5 g/L. The flasks were shaken at 75 rpm. 24 h continuous light, light intensity 20 μmol photons m^{-2} s^{-1}. Bars represent the standard deviation. n = 2.

At 22.5°C and 26°C, it is observed that the depletion of nitrate from MBL medium did not affect the growth of Bio 33. Difference in biomass between MBL medium with and without nitrate was not significant ($P < 0.05$). In conclusion, nitrate had no effect on the amount of biomass of Bio 33 in both media at 22.5°C and 26°C.

Table III-13. The specific growth rate μ and the mean doubling time G of strain Bio 33 grown in MBL medium with and without $NaNO_3$ at different temperatures (mean ± SD)

Temp. (°C)	Medium + 0.5% NaCl	μ (log_{10} unit per day)		G (days)	
		5 – 20 days	20 – 30 days	5 – 20 days	20 – 30 days
22.5	MBL	0.167 ± 0.032	0.058 ± 0.040	4.2 ± 0.8	15.7 ± 10.8
	MBL- N	0.140 ± 0.020	0.071 ± 0.030	5.0 ± 0.7	10.6 ± 4.4
26	MBL	0.135 ± 0.006	0.008 ± 0.000	5.1 ± 0.2	85.0 ± 4.9
	MBL- N	0.117 ± 0.004	0.025 ± 0.019	5.9 ± 0.2	39.9 ± 31.0

III.3.3. Effect of cobalt on growth of Bio 33

Another difference between BG 11 medium and MBL is the absence of the trace metal cobalt in BG 11 medium. In order to examine the influence of cobalt on growth of strain Bio 33, the strain was cultivated in BG 11 medium and MBL with and without cobalt.

III.3.3.1. *Bio 33 cultivated in BG 11 medium*

The growth of Bio 33 in BG 11 medium with cobalt recorded at different temperatures is represented in Figure III-24.

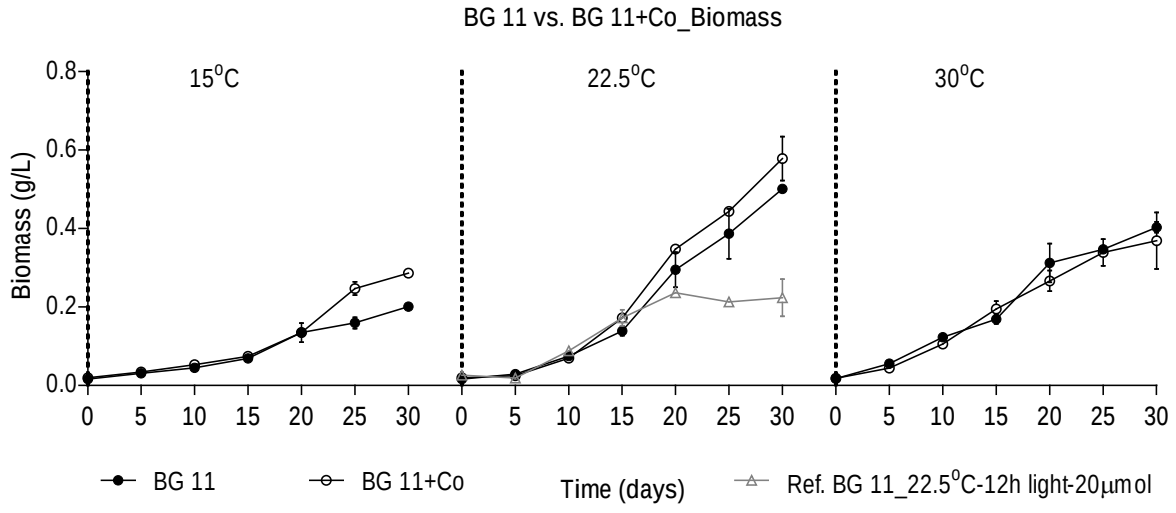


Figure III-24. Effect of cobalt on growth of Bio 33 in BG 11 medium.
Temperature: 15, 22.5 and 30°C. NaCl was added to all media at a concentration of 5 g/L. The flasks were shaken at 75 rpm. 24 h continuous irradiance, light intensity 20 μmol photons m^{-2} s^{-1}. Bars represent the standard deviation. n = 2.

The effect of cobalt on growth of Bio 33 changed according to temperature. At 15°C, cobalt slightly enhanced the growth of Bio 33. The effect was obvious after the 20th day. The difference in biomass

between medium with and without cobalt was significant ($P < 0.05$). At 22.5°C, the presence of cobalt in BG 11 medium enhanced the growth of Bio 33. The yield of biomass harvested after growing in BG 11 medium supplemented with cobalt was higher ($P < 0.05$). At 30°C, cobalt had no effect on the amount of biomass; difference was not significant ($P < 0.05$).

Table III-14. The specific growth rate μ and the mean doubling time G of strain Bio 33 grown in BG 11 medium with and without cobalt at different temperatures (mean ± SD)

Temp. (°C)	Medium + 0.5% NaCl	μ (log_{10} unit per day)		G (days)	
		5 – 20 days	20 – 30 days	5 – 20 days	20 – 30 days
15	BG 11	0.097 ± 0.018	0.041 ± 0.020	7.3 ± 1.3	19.1 ± 9.1
	BG 11+ Co^{2+}	0.092 ± 0.009	0.075 ± 0.007	7.6 ± 0.7	9.3 ± 0.8
22.5	BG 11	0.155 ± 0.001	0.054 ± 0.013	4.5 ± 0.0	13.4 ± 3.3
	BG 11+ Co^{2+}	0.176 ± 0.008	0.051 ± 0.012	4.0 ± 0.2	14.1 ± 3.4
30	BG 11	0.115 ± 0.008	0.026 ± 0.019	6.1 ±0.4	36.5 ± 27.0
	BG 11+ Co^{2+}	0.120 ± 0,012	0.032 ± 0.010	5.8 ± 0.6	22.9 ± 7.0

III.3.3.2. *Bio 33 cultivated in MBL medium*

The absence of cobalt in MBL medium at 15°C and 30°C did not affect the growth of Bio 33. The difference in biomass between medium with and without cobalt was not significant ($P < 0.05$). However, the depletion of cobalt from MBL medium at 22.5°C decreased the growth of Bio 33. Less amount of biomass was harvested from cultures grown in MBL medium without cobalt at 22.5°C ($P < 0.05$).

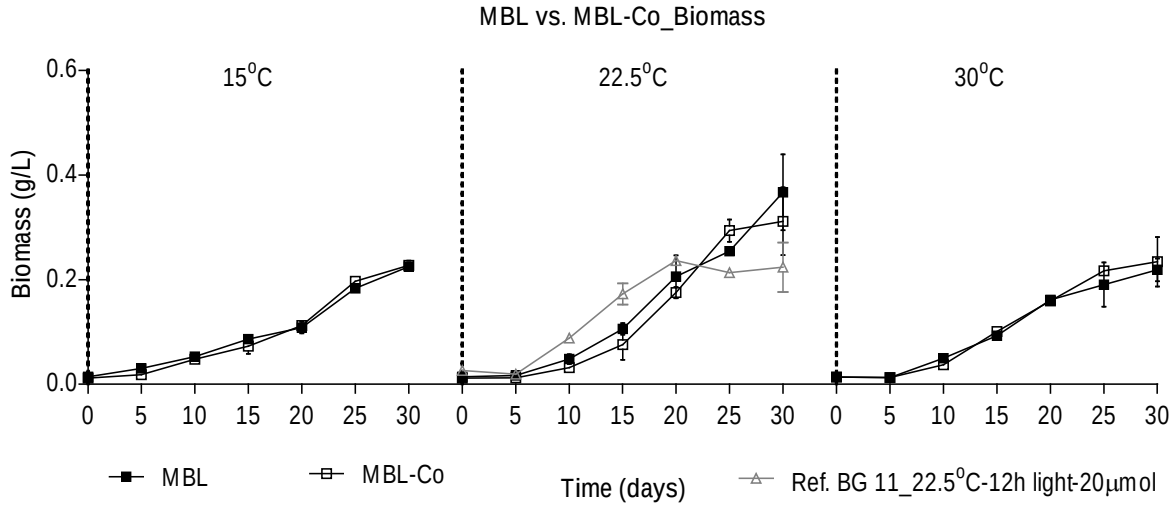


Figure III-25. Effect of cobalt on growth of Bio 33 in MBL medium.
Temperature: 18, 22.5 and 26°C, 24 h continuous irradiance, light intensity 20 μmol photons m^{-2} s^{-1}, shaking at 75 rpm. n = 2.

Table III-15. The specific growth rate μ and the mean doubling time of strain Bio 33 grown in MBL medium without cobalt at different temperatures (mean ± SD)

Temp. (°C)	Medium + 0.5% NaCl	μ (log_{10} unit per day)		G (days)	
		5 – 20 days	20 – 30 days	5 – 20 days	20 – 30 days
15	MBL	0.085 ± 0.014	0.073 ± 0.008	8.2 ± 1.3	9.5 ± 1.1
	MBL – Co^{2+}	0.122 ± 0.017	0.071 ± 0.009	5.7 ± 0.8	9.9 ± 1.3
22.5	MBL	0.167 ± 0.032	0.058 ± 0.040	4.2 ± 0.8	15.7 ± 10.8
	MBL – Co^{2+}	0.179 ± 0.012	0.057 ± 0.027	3.9 ± 0.3	13.8 ± 6.6
30	MBL	0.170 ± 0.013	0.030 ± 0.011	4.1 ± 0.3	24.8 ± 9.3
	MBL – Co^{2+}	0.168 ± 0.009	0.037 ± 0.020	4.1 ± 0.2	21.6 ± 11.6

In conclusion, supplementation of cobalt enhanced the growth of Bio 33 in both BG 11 and MBL media at 22.5°C. At lower and higher temperatures, no remarkable effect of cobalt on growth of Bio 33 was observed.

III.3.4. Effect of light on growth of Bio 33

The effect of light on growth of strain Bio 33 was studied in BG 11 and MBL medium with and without $NaNO_3$ (NaCl was added to all media at a concentration of 0.5%) at different light intensity and light period:

- 22.5°C, light/dark rhythm of 12 h/12 h, light intensity of 20 μmol photons m^{-2} s^{-1}
- 22.5°C, 24 h continuous light, light intensity of 20 μmol photons m^{-2} s^{-1} and,
- 22.5°C, 24 h continuous light, light intensity of 10 μmol photons m^{-2} s^{-1}.
- An enhancing of light intensity to 30 μmol photons m^{-2} s^{-1} and 24 h continuous light led to a bad growth of Bio 33 as the strain changed to yellow and died after exposing to the light some days. A light intensity over 40 μmol photons m^{-2} s^{-1} caused death to Bio 33.

The growth of strain Bio 33 cultivated in BG 11 plus 0.5% NaCl at 22.5°C, 20 μmol photons m^{-2} s^{-1}, light/dark rhythm of 12 h/12 h was used as reference. The results are shown in Figure III-26.

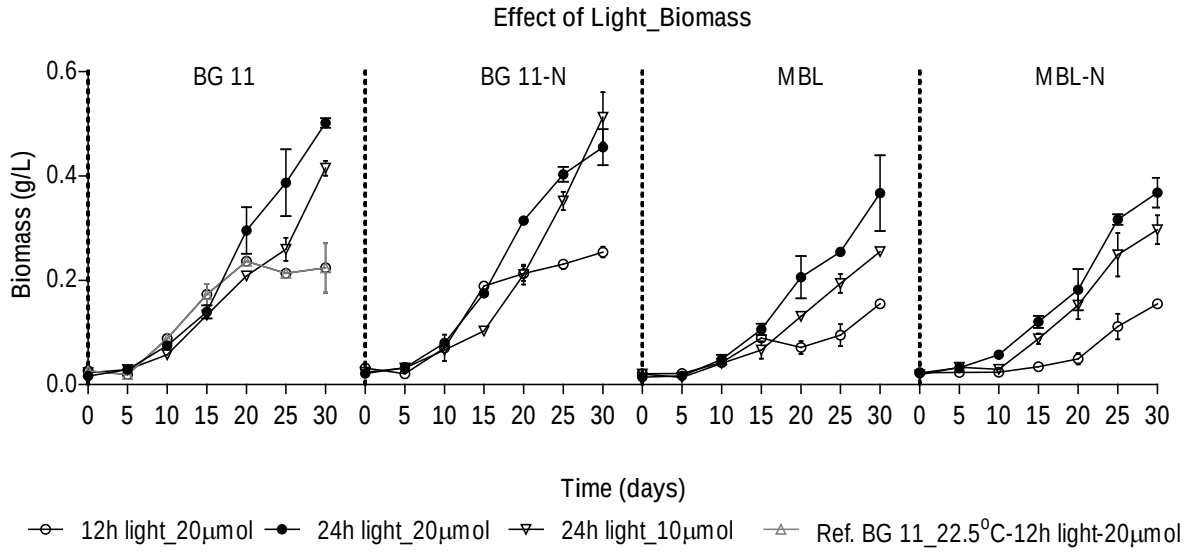


Figure III-26. Effect of light on growth of Bio 33.

Media: BG 11 with and without nitrate (BG 11 – N), MBL with and without nitrate (MBL – N). NaCl was added to all media at a concentration of 5 g/L. The flasks were shaken at 75 rpm, 22.5°C under different time of irradiance and light intensity: 1) 12 h light, light intensity 20 μmol photons m^{-2} s^{-1}; 2) 24 h continuous irradiance, light intensity 10 μmol photons m^{-2} s^{-1}; 3) 24 h light, light intensity 20 μmol photons m^{-2} s^{-1}. Bars represent the standard deviation. n = 2.

When Bio 33 was cultivated at 12 h/12 h light/dark rhythm under a light intensity of 20 μmol photons m^{-2} s^{-1}, the strain displayed higher growth rate in the log phase and reached the stationary phase at day 20th (Table III-16). However, when the time of irradiation was increased to 24 h continuous light, the strain continued growing after 20th day and had not reached the stationary phase at 30th day under both light intensities of 10 and 20 μmol photons m^{-2} s^{-1}. The yield of biomass harvested from the four tested media showed that, at the same light intensity of 20 μmol photons m^{-2} s^{-1}, the growth of Bio 33 was increased when the light/dark rhythm increased from 12 h light to 24 h light; difference in biomass was significant (one way ANOVA, $P < 0.001$). Besides, the growth of Bio 33 at 24 h of continuous light was better when the light intensity increased from 10 to 20 μmol photons m^{-2} s^{-1}; difference in biomass was significant (one way ANOVA, $P < 0.001$).

In conclusion, strain Bio 33 grew best at 24 h continuous light and light intensity of 20 μmol photons m^{-2} s^{-1} in all four media.

Table III-16. Specific growth rate μ and mean doubling time G of strain Bio 33 grown at 22.5°C in different media and light intensity (mean ± SD)

Medium + 0.5% NaCl	Time of irradiation (h) _Light intensity (μmol photons m^{-2} s^{-1})	μ (log_{10} unit per day)		G (days)	
		5 – 20 days	20 – 30 days	5 – 20 days	20 – 30 days
BG 11	12_20	0.168 ± 0.011	-0.007 ± 0.024	4.1 ± 0.3	18.4 ± 67.0
	24_10	0.133 ± 0.016	0.069 ± 0.004	5.3 ± 0.6	10.1 ± 0.6
	24_20	0.155 ± 0.001	0.054 ± 0.013	4.5 ± 0.0	13.4 ± 3.3
BG 11 - N	12_20	0.155 ± 0.006	0.018 ± 0.003	4.5 ± 0.2	39.6 ± 5.9
	24_10	0.127 ± 0.014	0.089 ± 0.000	5.5 ± 0.6	7.8 ± 0.0
	24_20	0.152 ± 0.005	0.037 ± 0.007	4.6 ± 0.1	19.2 ± 3.7
MBL	12_20	0.080 ± 0.022	0.078 ± 0.012	9.0 ± 2.5	9.0 ± 1.4
	24_10	0.149 ± 0.012	0.067 ± 0.003	4.7 ± 0.4	10.4 ± 0.5
	24_20	0.167 ± 0.032	0.058 ± 0.040	4.2 ± 0.8	15.7 ± 10.8
MBL - N	12_20	0.050 ± 0.025	0.116 ± 0.020	15.9 ± 7.9	6.1 ± 1.0
	24_10	0.101 ± 0.000	0.067 ± 0.009	6.9 ± 0.0	10.4 ± 1.3
	24_20	0.140 ± 0.020	0.071 ± 0.030	5.0 ± 0.7	10.6 ± 4.4

III.3.5. Effect of temperature on growth of Bio 33

III.3.5.1. *Effect of temperature in BG 11 medium*

To study the influence of temperature on growth of Bio 33 in BG 11 medium, the strain was cultivated at 15, 18, 22.5, 26 and 30°C. The specific growth rate μ and the doubling time G are represented in Table III-17.

Table III-17. The specific growth rate μ and the mean doubling time G of strain Bio 33 grown in BG 11 medium at different temperatures

Medium + 0.5% NaCl	Temp. (°C)	μ (log_{10} unit per day)		G (days)	
		5 – 20 days	20 – 30 days	5 – 20 days	20 – 30 days
BG 11	15	0.097 ± 0.018	0.041 ± 0.020	7.3 ± 1.3	19.1 ± 9.1
	18	0.106 ± 0.002	0.036 ± 0.005	6.5 ± 0.1	19.7 ± 3.0
	22.5	0.155 ± 0.001	0.054 ± 0.013	4.5 ± 0.0	13.4 ± 3.3
	26	0.120 ± 0.019	0.045 ± 0.028	5.8 ± 0.9	19.3 ± 12.2
	30	0.115 ± 0.008	0.026 ± 0.019	6.1 ±0.4	36.5 ± 27.0

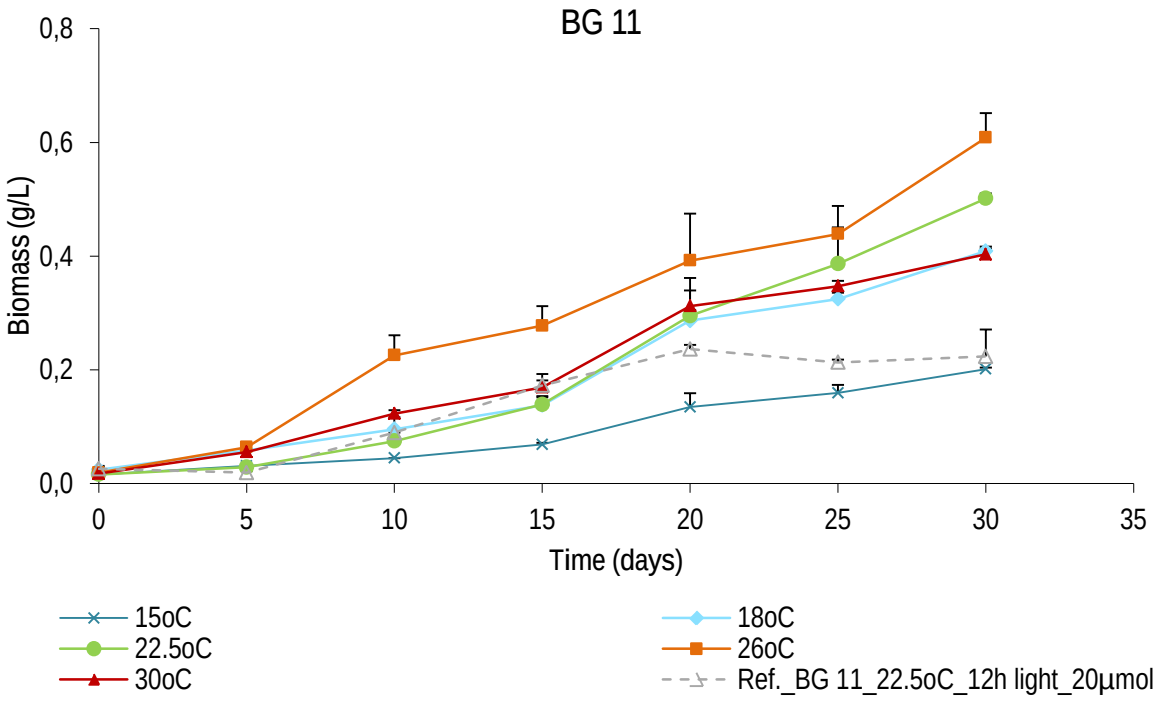


Figure III-27. Effect of temperature on growth of Bio 33 in BG 11 medium.

Temperature: 15, 18, 22.5, 26 and 30°C. NaCl was added at a concentration of 5 g/L. The flasks were shaken at 75 rpm, 24 h continuous light, light intensity 20 μmol photons m^{-2} s^{-1}. Bars represent the standard deviation. n = 2.

Bio 33 grew best in BG 11 medium at 26°C. Difference in biomass was significant (one way ANOVA, $P < 0.0001$). It is observed that Bio 33 had not reached the stationary phase at the 30th day. Based on the amount of biomass, from 15°C to 30°C, the growth of Bio 33 increased gradually with higher temperature up to a maximum growth at 26°C then decreased. The maximum amount of biomass was harvested at the 30th day at 26°C and was 3 fold higher than at 15°C. Hence, 26°C was considered as an optimum for growth of Bio 33 in BG 11 medium.

III.3.5.2. *Effect of temperature on growth of Bio 33 in MBL medium*

Strain Bio 33 was cultivated in MBL medium at 15, 22.5, 26 and 30°C. The same phenomenon as in BG 11 medium was observed. The yield of biomass was highest at 26°C. Difference in biomass was significant (one way ANOVA, $P < 0.001$). The growth of Bio 33 was proportional to the increasing of temperature from 15°C, reached an optimum at 26°C, and then decreased. The growth curves are represented in Figure III-28.

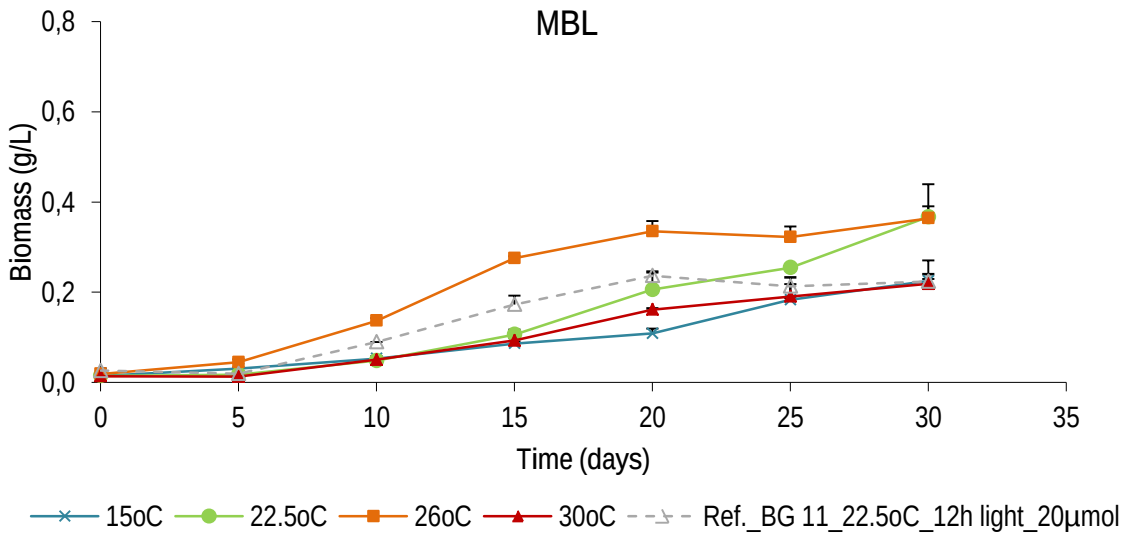


Figure III-28. Effect of temperature on growth of Bio 33 in MBL medium.

Temperature: 15, 22.5, 26 and 30°C. NaCl was added at a concentration of 5 g/L. The flasks were shaken at 75 rpm, 24 h continuous light, light intensity 20 μmol photons m^{-2} s^{-1}. Bars represent the standard deviation. n = 2.

Table III-18. The specific growth rate μ and the mean doubling time of strain Bio 33 grown in MBL medium at different temperatures

Medium + 0.5% NaCl	Temp. (°C)	μ (log_{10} unit per day)		G (days)	
		5 – 20 days	20 – 30 days	5 – 20 days	20 – 30 days
MBL	15	0.085 ± 0.014	0.073 ± 0.008	8.2 ± 1.3	9.5 ± 1.1
	22.5	0.167 ± 0.032	0.058 ± 0.040	4.2 ± 0.8	15.7 ± 10.8
	26	0.135 ± 0.006	0.008 ± 0.000	5.1 ± 0.2	85.0 ± 4.9
	30	0.170 ± 0.013	0.030 ± 0.011	4.1 ± 0.3	24.8 ± 9.3

From these experiments we conclude that, in principle higher temperatures are more advantageous for biomass production of Bio 33. The best growth of strain Bio 33 and highest yield of biomass were recorded in BG 11 medium at 26°C. Further increasing of temperature to 30°C led to the decreasing of growth rate and lower yield of biomass.

III.4. Method development and validation for the quantification of balticidins A-C from Bio 33

The method development and validation focused on balticidin A, B, and C (peaks 1, 2, and 3) as signed in Figure III-29.

III.4.1. Method development

III.4.1.1. *HPLC analysis*

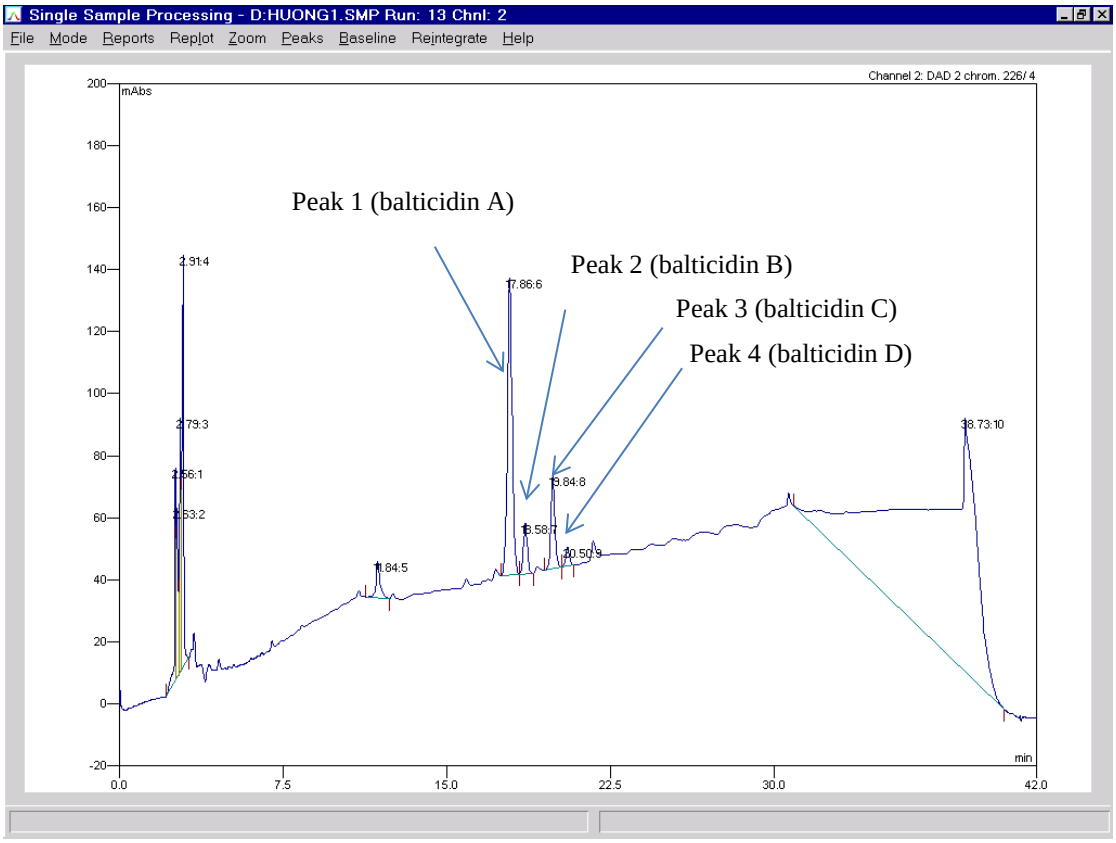


Figure III-29. Analyzing HPLC chromatogram of the 70% methanolic crude extract of strain Bio 33.

HPLC condition: Luna 5 μm C18 (2) 100 Å, 250 x 4.6 mm column (Phenomenex, USA), precolumn C18 (2), mobile phase MeOH/H_2O plus 0.05% TFA with mobile phase gradient in Table III-19, flow rate 1.0 mL min^{-1}, detection at 226 nm.

The best separation of the four peaks of the crude extract of strain Bio 33 (Figure III-29) was achieved with a Luna 5 μm C18 (2) 100 Å, 250 x 4.6 mm. (Phenomenex, USA) column with a guard column C18 (2). As mobile phase, a gradient of MeOH in water plus 0.05% TFA was used (HPLC program 2, see II.4.6.2). The flow rate was 1.0 mL min^{-1}. The column temperature was maintained at 25°C. Detection was performed at wavelength 226 nm. The injection volume was, typically, 40 μL. The concentration of the extract solution was equivalent to 30 mg of biomass in 2 mL solvent.

Table III-19. The mobile phase gradient for the analytical HPLC which was used for method development and validation experiments

Time (min)	0.0	0.5	4.5	7.5	30.5	35.5	37.5	42.5
% MeOH	40	40	60	70	100	100	40	40

III.4.1.2. *Effect of filter and solvent on the preparation of HPLC samples*

The MeOH/H_2O (50%) extract of strain Bio 33 was dissolved in MeOH/H_2O in different ratios (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9). Each sample was filtered with three different types of filter (cellulose acetate membrane, nylon and PTFE). The samples were subsequently analyzed by HPLC and 20 μL/run was injected. The area of peaks 1, 2 and 3 are shown in Figure III-30.

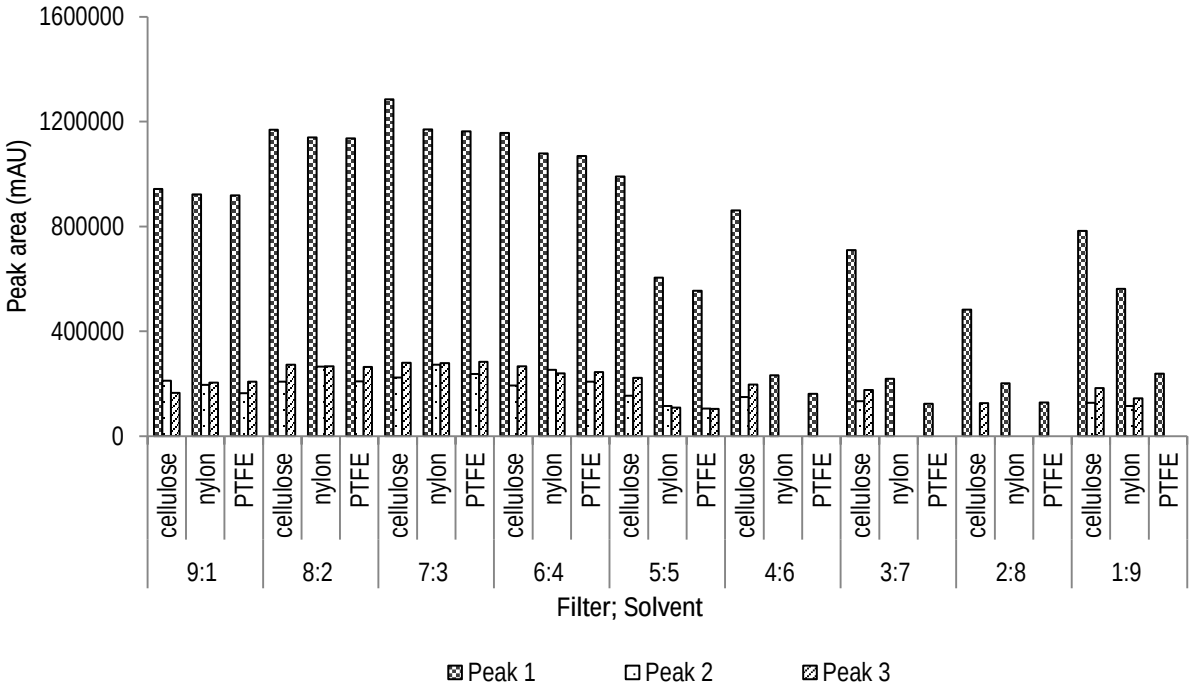


Figure III-30. Effect of solvent and filter on the content of peak 1, 2, and 3 in the crude extract MeOH/H_2O (50%).

The extract disolved in MeOH/H_2O (7:3) and filtered with a cellulose acetate membrane filter showed the highest area of all three peaks. As result, the cellulose acetate membrane filter (0.2 μm) and MeOH/H_2O (7:3) are used to prepare the HPLC samples.

III.4.1.3. *Effect of solvents on the extraction of the active compounds*

The same amount of biomass was extracted with different solvents. With each solvent, the biomass was extracted 3 times. The residues were extracted the fourth time with MeOH/H_2O (7:3). The percentage masses of the crude extract, namely E1, E2, E3, E4, are shown in Table III-20. The amount of crude extract was highest (30.69% in total) when the biomass was extracted with MeOH 50% in H_2O and lowest (16.79% in total) with MeOH 40% in H_2O.

All extracts were analyzed by HPLC and the areas of the main peaks 1, 2 and 3 are represented in Figure III-31. The solvents MeOH 100%, 90%, 80% were not strong enough to extract all active compounds in the first two extractions, so that they were concentrated in the third and fourth extract. The best results were shown with MeOH 70%. MeOH 60%, 50% and 40% extracted lower amounts of

active compounds. When EtOH 100% was used, the active compounds were not extracted in the first three extraction steps, so that the best extraction yield was achieved only by the fourth extraction with MeOH 70%. The amount of extract yielded with EtOH 70% was higher than with MeOH 70%, but the yield of active compounds was higher when the extracting solvent was MeOH 70%. From the above results, MeOH 70% in H_2O was recognized as the best extracting solvent for the active substances.

Table III-20. Percentage mass of the crude extracts extracted with different solvents.

No.	Extracting solvent 1	Extracting solvent 2	Extract	Extracted mass (%, ±SD)
1.	MeOH 100%		E1+E2	19.7±0.3
	MeOH 100%		E3	0.7±0.1
		MeOH 70%	E4	4.5±0.2
2.	MeOH 90%		E1+E2	20.9±4.8
	MeOH 90%		E3	1.1±0.1
		MeOH 70%	E4	2.8±0.2
3.	MeOH 80%		E1+E2	20.8±2.0
	MeOH 80%		E3	0.9±0.1
		MeOH 70%	E4	2.3±0.1
4.	MeOH 70%		E1+E2	21.5±0.2
	MeOH 70%		E3	1.9±2.1
		MeOH 70%	E4	2.2±1.0
5.	MeOH 60%		E1+E2	20.7±0.3
	MeOH 60%		E3	0.2±0.1
		MeOH 70%	E4	2.7±0.6
6.	MeOH 50%		E1+E2	27.3±1.2
	MeOH 50%		E3	0.6±0.4
		MeOH 70%	E4	2.8±0.2
7.	MeOH 40%		E1+E2	13.2±1.5
	MeOH 40%		E3	0.20±0.0
		MeOH 70%	E4	3.4±0.6
8.	EtOH 100%		E1+E2+E3	12.7±0.1
		MeOH 70%	E4	10.6±0.6
9.	EtOH 70%		E1+E2+E3	24.9±0.1
		MeOH 70%	E4	1.4±0.0
10.	EtOH 50%		E1+E2+E3	21.5±1.9
		MeOH 70%	E4	1.6±0.1
11.	MeOH 70%		E1+E2+E3	23.1±3.0
		MeOH 70%	E4	1.4±1.2

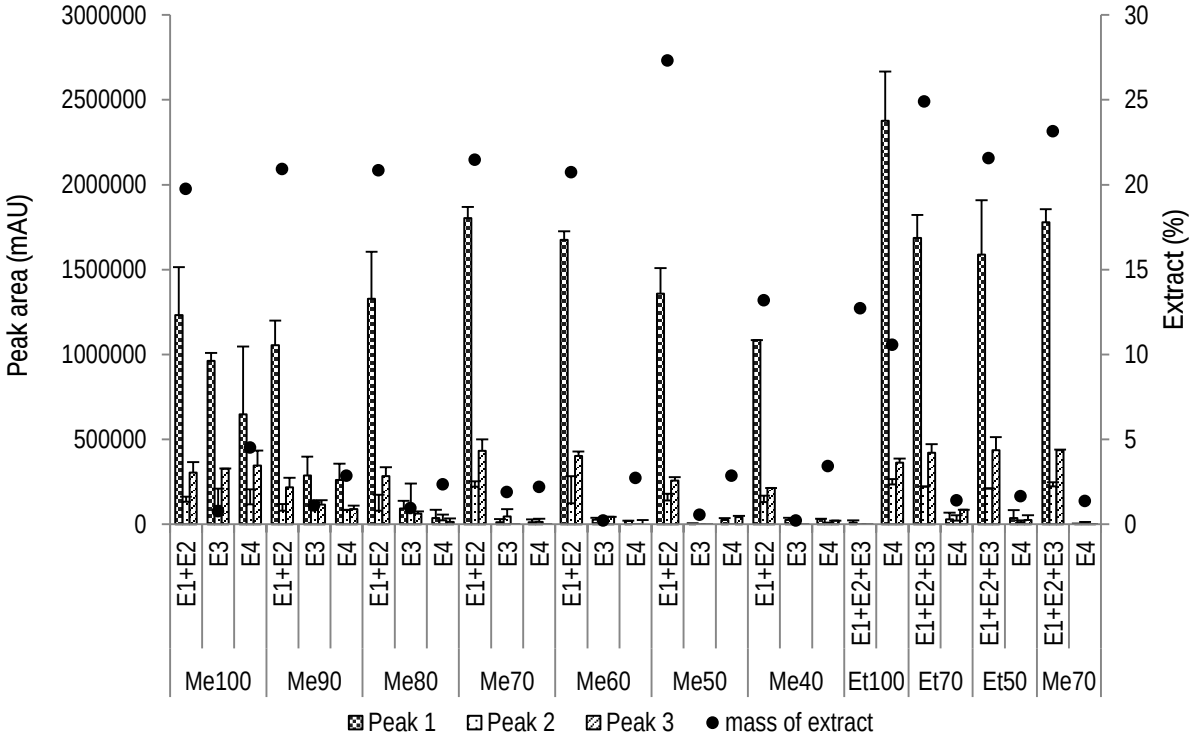


Figure III-31. Peak area of peak 1, 2 and 3 of the crude extracts extracted with different solvents.
E1+E2/E1+E2+E3 dissolved in the extracting solvent, concentration equivalent to 15 mg biomass/ 1 mL solvent.
E3 dissolved in 500 μL extracting solvent. E4 dissolved in 1 mL of MeOH/H_2O (7:3).

III.4.1.4. *Effect of extraction time and extraction volume*

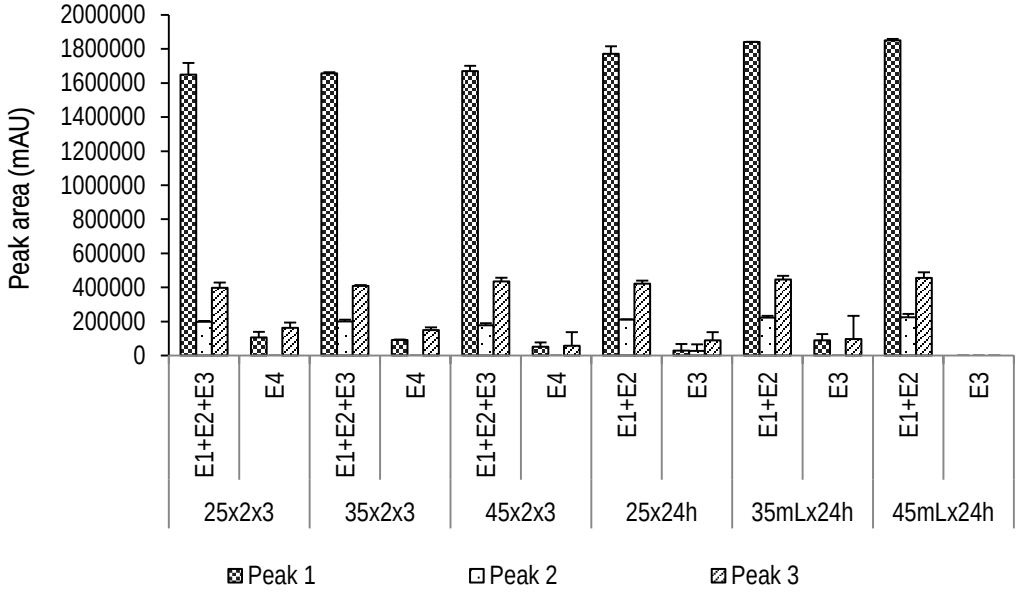


Figure III-32. Peak area of peak 1, 2 and 3 from the biomass extracted with different extraction time and extraction volume.

E1+E2+E3/E1+E2 was dissolved in the extracting solvent, the concentration was equivalent to 15 mg biomass in 1 mL solvent. E4/E3 was dissolved in 500 μL MeOH/H_2O (7:3).

The percentage mass of the crude extract is shown in Table III-29. The HPLC analysis of the peak areas is shown in Figure III-32. The highest concentration of peak 1, 2 and 3 was obtained when the extraction time and volume was 45 mL x 24 h followed by 45 mL x ½ h. Hence, this method was chosen to extract the active compounds from the biomass of Bio 33.

Table III-21. Influence of different extraction time and extraction volume on MeOH/H_2O (7:3) extracts.

No.	Volume of solvent/Extraction-time/number of extraction	Extract	Mass of extract (%, ±SD)
1.	25mL x 2h x 3times	E1+E2+E3	19.6±0.8
	25mL x ½h	E4	0.8±0.4
2.	35mL x 2h x 3times	E1+E2+E3	19.9±0.5
	35mL x ½h	E4	0.7±0
3.	45mL x 2h x 3times	E1+E2+E3	20.8±0.1
	45mL x ½h	E4	0.7±0
4.	(25mL x 24h)+(25mL x ½h)	E1+E2	20.8±0.2
	25mL x ½h	E3	0.7±0.1
5.	(35mL x 24h)+(35mL x ½h)	E1+E2	23.1±2.8
	35mL x ½h	E3	1.1±0.2
6.	(45mL x 24h)+(45mL x ½h)	E1+E2	23.1±0.04
	45mL x ½h	E3	0.6±0.1

III.4.1.5. *Effect of tubes used for extraction on quantification of active compounds*

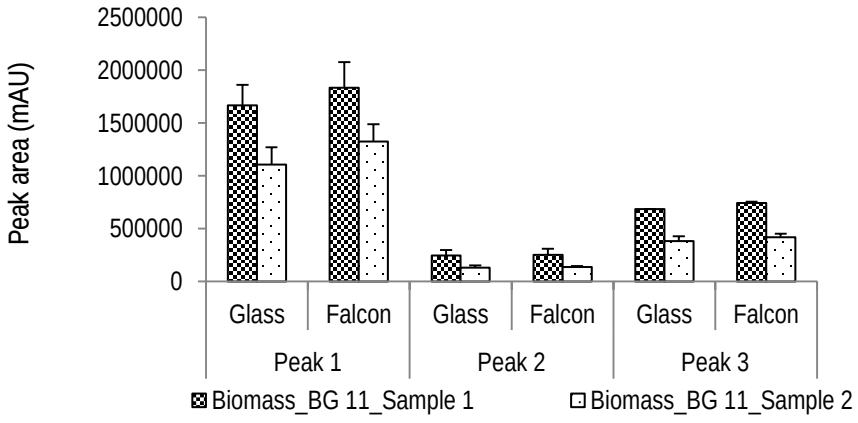


Figure III-33. Effect of tubes used for extraction on peak areas.
Extracting solvent MeOH/H_2O (7:3). Injection 40 μL/run.

The results showed that using 100 mL glass Erlenmeyer flasks to extract biomass led to a slightly reduction of peak areas in comparison to extraction in 50 mL Falcon tubes. The same result was recorded using different biomasses. Therefore, Falcon tubes were chosen for extracting the biomass of strain Bio 33.

III.4.1.6. *Effect of ultrasound and position of the extraction tube*

As recorded, the temperature of the ultrasonic bath was around 40°C after 60 min of sonication (Figure III-36). Hence, the water in the ultrasonic bath was renewed every hour. The effect of ultrasound on the stability of the peaks was studied. No considerable differences in peak shape and peak area were observed in samples sonicated for 0, 15, 30 and 60 min in vertical Falcon.

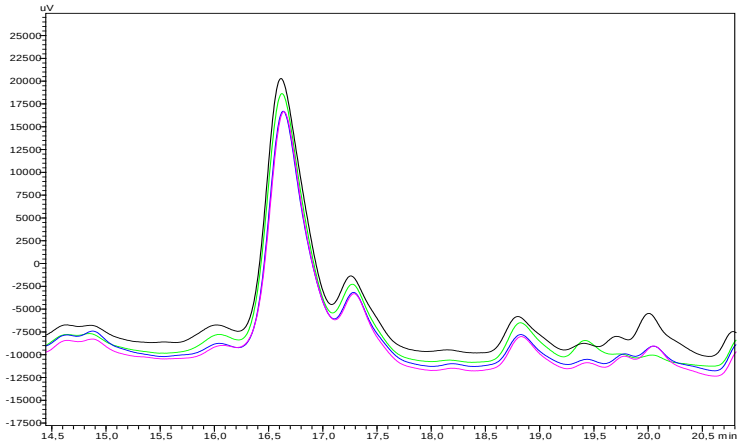


Figure III-34. Effect of sonication on the stability of active compounds.

Overlay chromatograms of samples sonicated for 5 min (black), 15 min (pink), 30 min (blue), 60 min (green). Biomass was extracted with MeOH/H_2O (7:3). Injection 20 μL/run. Concentration equivalent to 15 mg biomass/mL solvent.

Another experiment on the effect of ultrasound was carried out with biomass extracted with MeOH/H_2O (1:1). The HPLC results showed that after 2 h or longer time of sonication, the peaks were deformed (see Figure III-35).

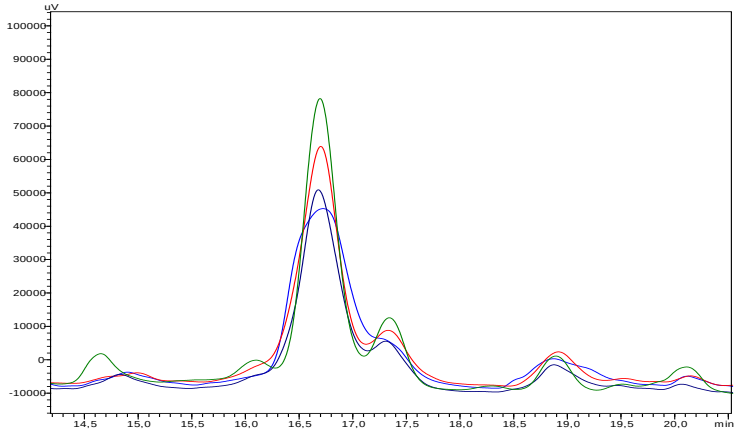


Figure III-35. Effect of sonication on the stability of active compounds.

Overlay chromatograms of samples sonicated for 60 min (green), 2 h (red), 6 h (navy), 12 h (blue). Biomass was extracted with MeOH/H_2O (1:1). Injection 40 μL/run. Concentration equivalent to 15 mg biomass/mL solvent

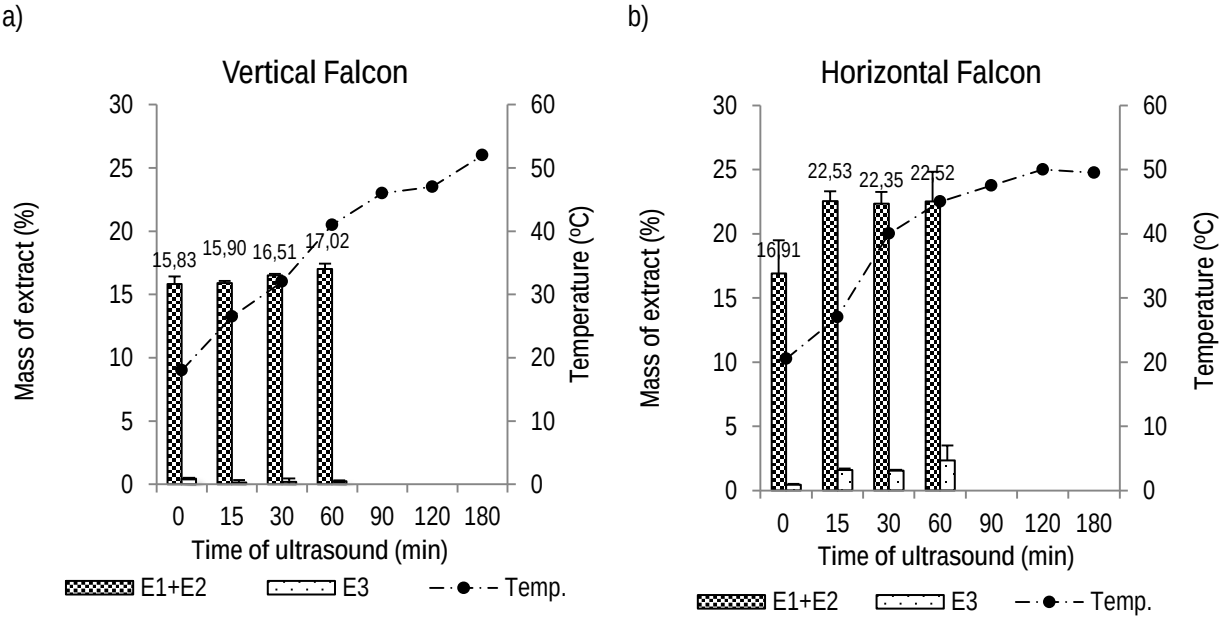


Figure III-36. Effect of ultrasound on the percentage mass of the crude extracts.

Sonification of 0, 15, 30, 60 min with a) vertical Falcon, b) horizontal Falcon.

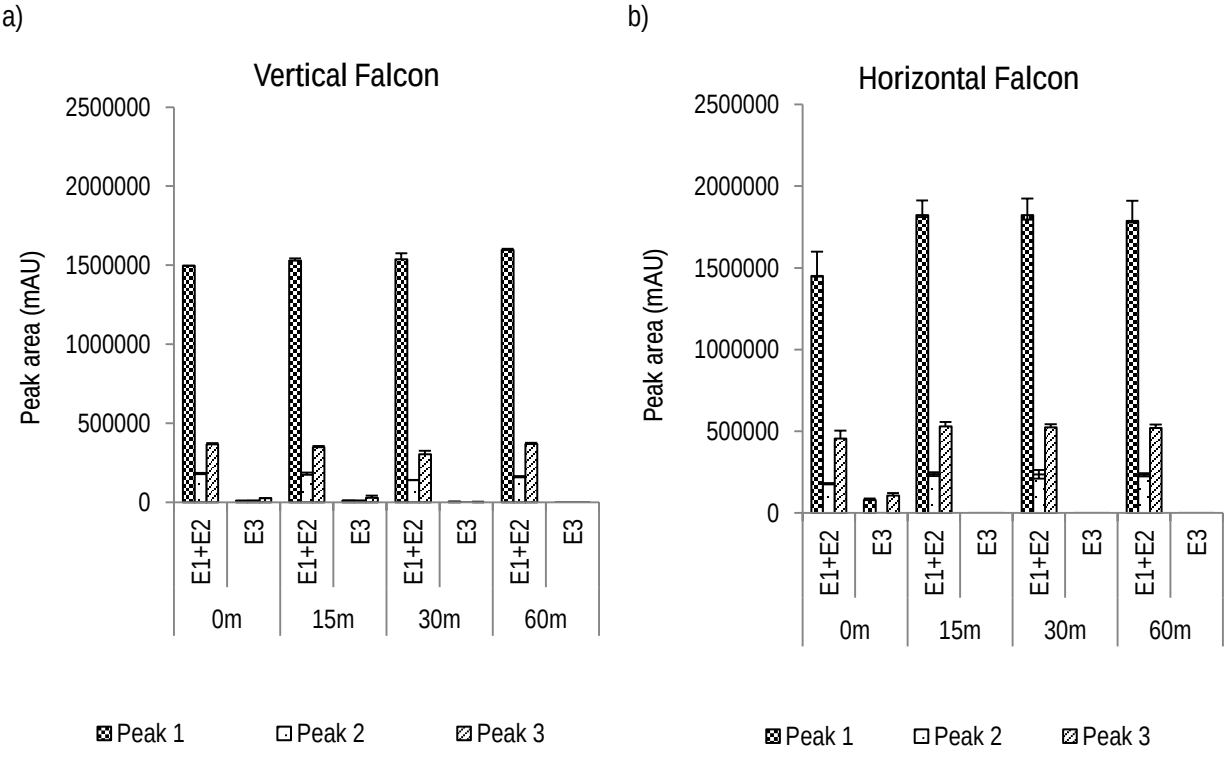


Figure III-37. Effect of ultrasound on the peak areas of 70% MeOH extracts.

Sonication 0, 15, 30, 60 min with a) vertical Falcon, b) horizontal Falcon. E1+E2 dissolved in MeOH 70%, concentration was equivalent to 15 mg biomass/1 mL extracting solvent. E3 dissolved in 500 μL of extracting solvent. Injection 40 μL/run.

When the tubes were placed horizontally in the ultrasonic bath, the extract mass and peak area of active compounds increased about 25% in comparison to that of the vertical placed tubes. Fifteen

minutes of sonication were sufficient for the extraction. When the sonication time was increased the yield of extracts as well as active compounds were not enhanced. As shown in Figure III-37, in the range of test temperature (< 45°C), the yield of extract as well as the peak area was not effected by temperature.

III.4.1.7. *Evaluation of the extraction conditions*

The amount of extract collected after each extraction was shown in Table III-22. Most of the crude extract was detected in the first 45 mL solvent.

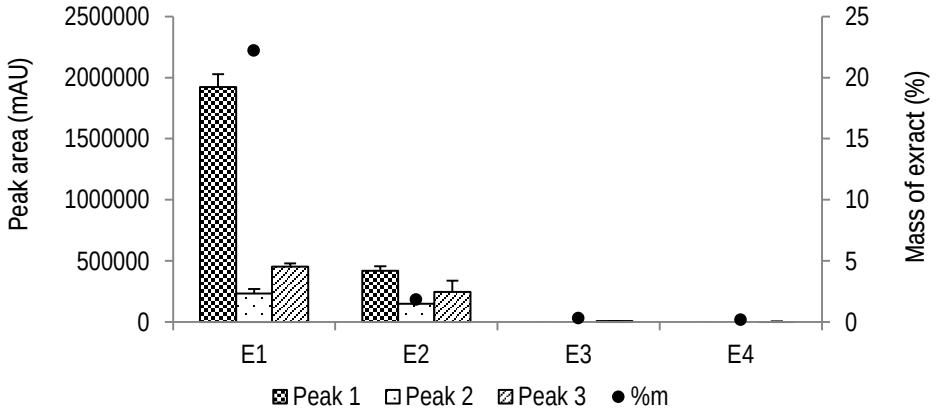


Figure III-38. Area of peak 1, 2 and 3 in crude extracts of stepwise extraction.

E1 = 1st, E2 = 2nd; E3 = 3rd; E4 = 4th extraction. E1 dissolved in 70% MeOH. Concentration equivalent to 15 mg biomass/mL. E2, E3, E4 dissolved in 500 μL. Injection 40 μL/run.

Table III-22. Percentage mass of 70% MeOH extracts extracted with different time periods

Volume of solvent (mL)	Time of extraction	Name of extract	Extracted mass (%, ±SD)
45	24h	E1	24.2±1.9
45	½h	E2	1.8±1.1
45	½h	E3	0.3±0.2
45	½h	E4	0.2±0.1

The analytical HPLC showed that the active compounds were extracted completely from the biomass in two steps with 45 mL MeOH/H_2O (7:3)

In summary, the following optimized method for quantification of peak 1, 2 and 3 was developed:

The dried biomass was extracted with 45 mL of MeOH/H_2O (7:3) contained in a 50 mL Falcon tube. The tube was placed horizontally in the ultrasonic bath for 15 min. After that, the biomass was extracted by the magnetic stirrer (1000 rpm, stirrer bar 15 x 5 mm) for 24 h. After extracting, the tube was centrifuged at 4300 rpm for 7 min and the supernatant E1 was filtered with Whatman filter paper

(Grade 1, 11 μm). The residue was extracted again with 45 mL MeOH/H_2O (7:3) for ½ h. After centrifugation the supernatant E2 was filtered and combined with E1. The supernatants were dried in rotary evaporator and lyophilized. To analyze the active compounds, MeOH/H_2O (7:3) was added to the crude extract to get a concentration equivalent to 15 mg biomass/1 mL solvent. The sample was sonicated for 15 min to dissolve all the active compounds. The sample was filtered with a cellulose acetate membrane filter (0.2 μm, 25 mm) and 40 μL were loaded on a Luna 5 μm C18 (2) (100 Å, 250x4.6 mm, Phenomenex, USA) column with a guard column C18 (2). As mobile phase MeOH/H_2O plus 0.05% TFA with a flow rate of 1.0 mL min^{-1} was used. The chromatogram was detected at 226 nm. The mobile phase gradient for the analytical HPLC was the running program 2:

Time (min)	0.0	0.5	4.5	7.5	30.5	35.5	37.5	42.5
% MeOH	40	40	60	70	100	100	40	40

III.4.2. Method validation for quantification of balticidins A-C

III.4.2.1. *Method reproducibility*

In the repeatability study the percent relative standard deviations (% RSD) for retention time and peak area of peak 1 were 0.1 and 0.3%, better than for peak 2 (0.2 and 0.8%) and peak 3 (0.1 and 2.9%) (Table III-26). The estimation of intermediate precision showed the same order of magnitude as for repeatability study:% RSD values for retention time and peak area of peak 1 were 0.2 and 0.5%, better than for peak 2 (0.3 and 2.6%) and peak 3 (0.2 and 3.8%).

III.4.2.2. *Linearity*

Good linearity was observed over the concentration range equivalent to 0.75 - 15 mg biomass/mL (n = 6). The linear regression equation for peak 1 was $y_1 = 121498x + 5349.4$ (correlation coefficient $R^2 = 0.9993$), for peak 2 $y_2 = 14312x + 2672.1$ ($R^2 = 0.9947$), and for peak 3 $y_3 = 26637x + 16903$ ($R^2 = 0.9989$). The percentage relative standard deviation of the slope and *Y*-intercept of the calibration plot for peak 1 were 0.1 and 12.0%, for peak 2 were 0.5 and 14.8% and for peak 3 were 0.4 and 6.4% (Table III-26).

The content of compound 4 in the control biomass (concentration equivalent to 15 mg biomass/mL) was too low to quantify (LOD ~ 11 mg biomass/mL and LOQ ~ 33 mg biomass/mL). Moreover, HPLC analysis confirmed that there was an overlap of peak 4 with other compounds in the 70% MeOH crude extract. Consequently, the quantification was done only for peak 1, peak 2 and peak 3.

III.4.2.3. *LOD and LOQ*

The limits of detection (LOD) and quantification (LOQ) were estimated for peak 1 as 0.5 and 1.6 mg biomass/mL, for peak 2 as 1.4 and 4.4 mg biomass/mL, for peak 3 as 0.6 and 1.9 mg biomass/mL, respectively. Method precision for peak 1, peak 2 and peak 3 at the limit of quantification was 9.8, 7.9 and 9.4% RSD (Table III-26).

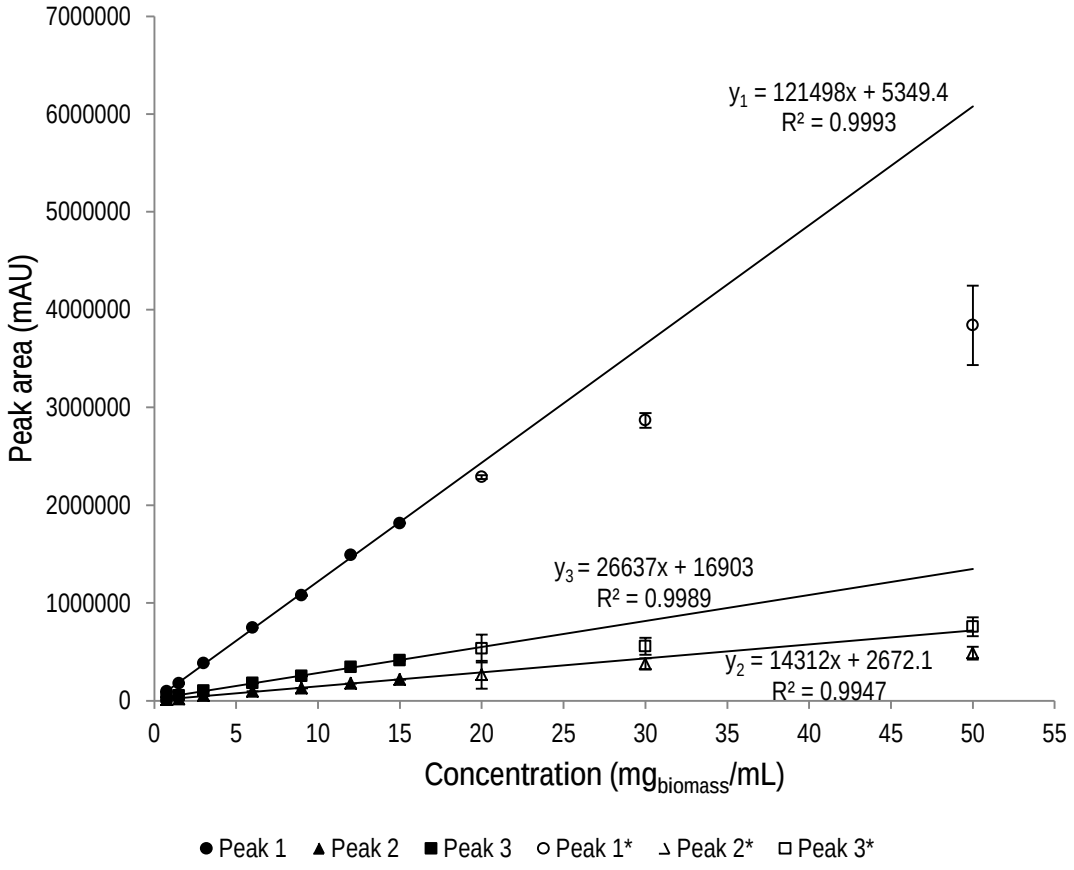


Figure III-39. Calibration curves.

"*": points from recovery experiment (see III.4.2.4), out of the calibration curves.

III.4.2.4. *Accuracy and recovery*

2, 4, 8, 15, 30, 40, 60 and 100 mg of dried biomass were extracted according to the protocol described in III.4.1.7 (n = 3). The crude extracts were dissolved in 2 mL MeOH/H_2O (7:3). 40 μL of each were loaded on the analytical HPLC column. The areas of the peaks were determined. The recoveries of peak 1, 2 and 3 were calculated from the slope and *Y*-intercept of the calibration plot obtained in III.4.2.2. The recovery suggested that the reliable concentration of sample prepared for HPLC analysis should not be higher than 15 mg biomass/mL solvent. The peak area-responses of concentrations equivalent to 20, 30, 50 mg biomass/mL are presented in Figure III-39 (peak 1*, 2*, 3*).

Table III-23. Recovery of biomass according to peak 1.

The linear regression equation y_1 = 121498x + 5349.4			
C_{known} (mg biomass/mL)	Peak 1 area (±SD)	$C_{estimated}$ (±SD) (mg biomass /mL)	Recovery (%) (±% RSD)
1	117744.5 ± 12824	0.93 ± 0.11	92.51 ± 11.41
2	241632 ± 16453	1.94 ± 0.14	97.24 ± 6.96
4	521547 ± 5222	4.25 ± 0.04	106.22 ± 1.01
7.5	884359 ± 9994	7.23 ± 0.08	96.46 ± 1.14
15	1818822 ± 1606	14.93 ± 0.01	99.51 ± 0.09
20	2288125 ± 19684	18.79 ± 0.16	93.94 ± 0.86
30	2867205 ± 76065	23.55 ± 0.63	78.52 ± 2.66
50	3839672 ± 406615	31.56 ± 3.35	63.12 ± 10.60

Table III-24. Recovery of biomass according to peak 2.

The linear regression equation y_2 = 14312x + 2672.1			
C_{known} (mg biomass/mL)	Peak 2 area (±SD)	$C_{estimated}$ (±SD) (mg biomass/mL)	Recovery (%) (±% RSD)
1	15964 ± 1528	0.93 ± 0.11	92.87 ± 11.49
2	32029 ± 1018	2.05 ± 0.07	102.56 ± 3.47
4	65994 ± 2773	4.42 ± 0.19	110.61 ± 4.38
7.5	115549 ± 5000	7.89 ± 0.35	105.16 ± 4.43
15	222082 ± 5550	15.33 ± 0.39	102.20 ± 2.53
20	265375 ±142742	18.36 ± 1.23	91.78 ± 6.71
30	376754 ± 58093	26.14 ± 4.06	87.13 ± 15.53
50	486609 ± 66764	33.81 ± 4.66	67.63 ± 13.80

Table III-25. Recovery of biomass according to peak 3.

The linear regression equation y_3 = 26637x + 16903			
C_{known} (mg biomass/mL)	Peak 3 area (±SD)	$C_{estimated}$ (±SD) (mg biomass/mL)	Recovery (%) (±% RSD)
1	43126 ± 2985	0.98 ± 0.11	98.45 ± 11.39
2	72305 ± 2721	2.08 ± 0.11	103.99 ± 4.91
4	130235 ± 9802	4.25 ± 0.37	106.37 ± 8.65
7.5	213264 ± 5551	7.37 ± 0.21	98.29 ± 2.83

15	423009 ± 3159	15.25 ± 0.12	101.64 ± 0.78
20	534311 ± 142742	19.42 ± 0.44	97.12 ± 2.28
30	556883 ± 85440	20.27 ± 3.21	67.57 ± 15.84
50	755421 ± 96225	27.73 ± 3.61	55.45 ± 13.03

III.4.2.5. *Stability in solution*

The overall relative standard deviation of the peak area was calculated for all injections (3 times per day over three days).% RSD values of peak area were 3.5, 6.4 and 5.3 for peak 1, 2 and 3, respectively.% RSD values of retentiontime were 0.18, 0.20 and 0.15 for peak 1, 2 and 3, respectively. These values were higher than the values recorded for intermediate precision (between days, see III.4.2.1). The results showed that the HPLC solutions of the crude extract of strain Bio 33 in MeOH/H_2O (7:3) (concentration equivalent to 15 mg biomass/mL) placed in the autosampler were stable for at least 48 h. Besides, the sample (crude extract in MeOH/H_2O (7:3) concentration equivalent to 15 mg biomass/mL) precipitated after 2 days stored at 4°C. This fact indicated that the sample should be prepared and analyzed freshly.

Table III-26. Results of method validation

Property	Peak 1	Peak 2	Peak 3
Repeatability (n = 6,% RSD)			
Retention time (t_R)	0.1	0.2	0.1
Peak area	0.3	0.8	2.9
Intermediate (between-days) precision (n =12,% RSD)			
Retention time (t_R)	0.2	0.3	0.2
Peak area	0.5	2.6	3.8
Linearity (n = 6)			
Calibration range (~ mg biomass/mL)	0.75 - 15	0.75 - 15	0.75 - 15
Calibration points	7	7	7
Correlation coefficient	0.9993	0.9947	0.9989
Slope (%RSD)	0.1	0.5	0.4
Intercept (% RSD)	12.0	14.8	6.4
LOD (~ mg biomass/mL)	0.5	1.4	0.6
LOQ (~ mg biomass/mL)	1.6	4.4	1.9
Precision (at LOQ, n = 5)	9.8	7.9	9.4

Stability in solution (3 replicates, over 3 days,% RSD)			
Retention time (t_R)	0.18	0.20	0.15
Peak area	3.5	6.4	5.3

III.5. Production of balticidins A-C of strain Bio 33

The increasing or decreasing in the peak area of the three compounds (FIII-4, FIII-5, FIII-6) recorded under different cultivating conditions reflected the effect of cultivating conditions on the production of the active compounds. The production of the bioactive compounds of strain Bio 33 cultivated in BG 11 medium plus 0.5% NaCl at 22.5°C, 20 μmol photons m^{-2} s^{-1}, 12 h/12 h light/dark rhythm was used as reference.

The method used for investigation the production of active compounds (peak 1, 2, 3) of Bio 33 from the growth curves (biomass range from 2 mg to 100 mg) was described in III.4.1.7.

III.5.1. Effect of culture medium on the production of balticidins A-C

It was obverved that the effect of culture media on the production of balticidins varied with temperature (time of irradiance and light intensity were fixed at 24 h continuous light and 20 μmol photons m-2 s-1 , respectively (Figure III-40)). The difference in the area of peak 1, 2 and 3 between BG 11 and MBL media was significant ($P < 0.05$). In particularly, at 15°C, Bio 33 cultivated in MBL medium produced more balticidins B (peak 2) and C (peak 3) than in BG 11 medium. However, at 22.5°C and higher temperatures, Bio 33 cultivated in MBL produced less balticidins B and C than in BG 11.

In case of balticidin A (peak 1), the area of peak 1 produced by Bio 33 cultivated in MBL medium was higher than that in MBL medium at 15°C, 26°C and 30°C. At 22.5°C, the mean of peak 1 area (mean of all determinations) of Bio 33 cultivated in BG 11 medium was highest, but the highest concentration of peak 1 was detected at 30th day in biomass cultivated in MBL medium at 22.5°C. At 22.5°C, the production of peak 1 in BG 11 medium increased quickly from 5th day to 10th day and became stable after 15th day; in contrast, in MBL medium the concentration of peak 1 still increased until 30th day of cultivation.

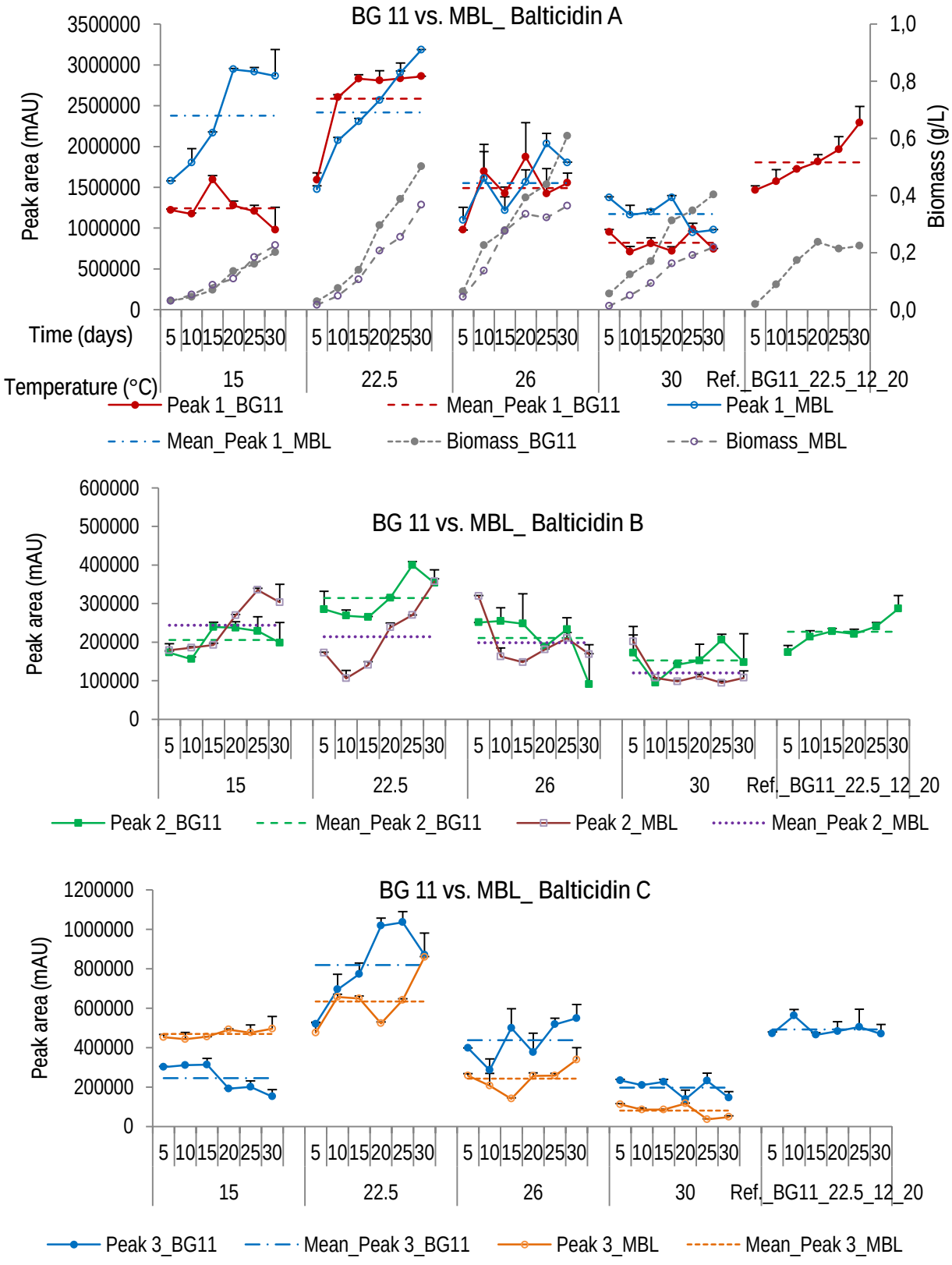


Figure III-40. Effect of BG 11 and MBL media on balticidins A-C.

Discontinuous straight lines: mean of balticidin concentration over the cultivation time. The error bars represent the standard deviation. n = 2.

According to the high yield of biomass and high concentration of balticidins A, B, C of Bio 33 cultivated in BG 11 medium, BG 11 medium was considered as the better medium for an effective production of balticidins A, B and C, especially at 22.5°C.

III.5.2. Effect of nitrate on the production of balticidins A-C

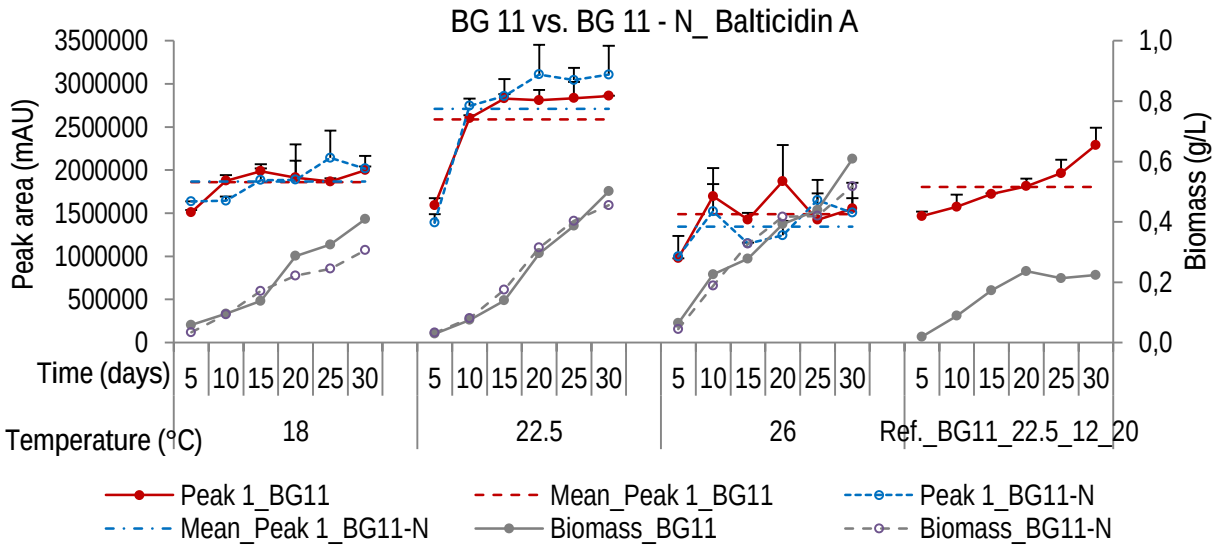
III.5.2.1. *Strain Bio 33 cultivated in BG 11 medium*

The influence of nitrogen depletion on the production of balticidins was carried out in BG 11 medium at 18, 22.5, and 26°C, under 24 h continuous irradiance with a light intensity of 20 μmol photons m^{-2} s^{-1}, and a shaking frequency of 75 rpm. The analytical HPLC of antifungal lipopeptide concentration is represented in Figure III-41.

Peak 1: When Bio 33 was cultivated under 24 h of continuous irradiance and a light intensity of 20 μmol photons m^{-2} s^{-1}, it was observed that nitrate had no effect on the production of peak 1. There were no significant differences in the area of peak 1 ($P < 0.05$) at all tested temperatures. The concentration peak 1 was nearly identical in BG 11 medium with and without nitrate.

Peak 2: At 22.5°C, Bio 33 produced more balticidin B in BG 11 medium without nitrate ($P < 0.05$) in comparison to BG 11 medium with nitrate. However, the depletion of nitrate from BG 11 medium at other tested conditions did not have significant effect on the production of peak 2. The difference in the area of peak 2 between BG 11 medium with and without nitrate was not significant ($P < 0.05$).

Peak 3: At all tested temperatures, the depletion of nitrate from BG 11 medium reduced the production of peak 3; difference in peak areas was significant ($P < 0.0001$). In conclusion, at 22.5°C - when the production of balticidins was highest - the depletion of nitrate from BG 11 medium did not affect the production of balticidin A but increased the production of balticidin B and decreased the production of balticidin C.



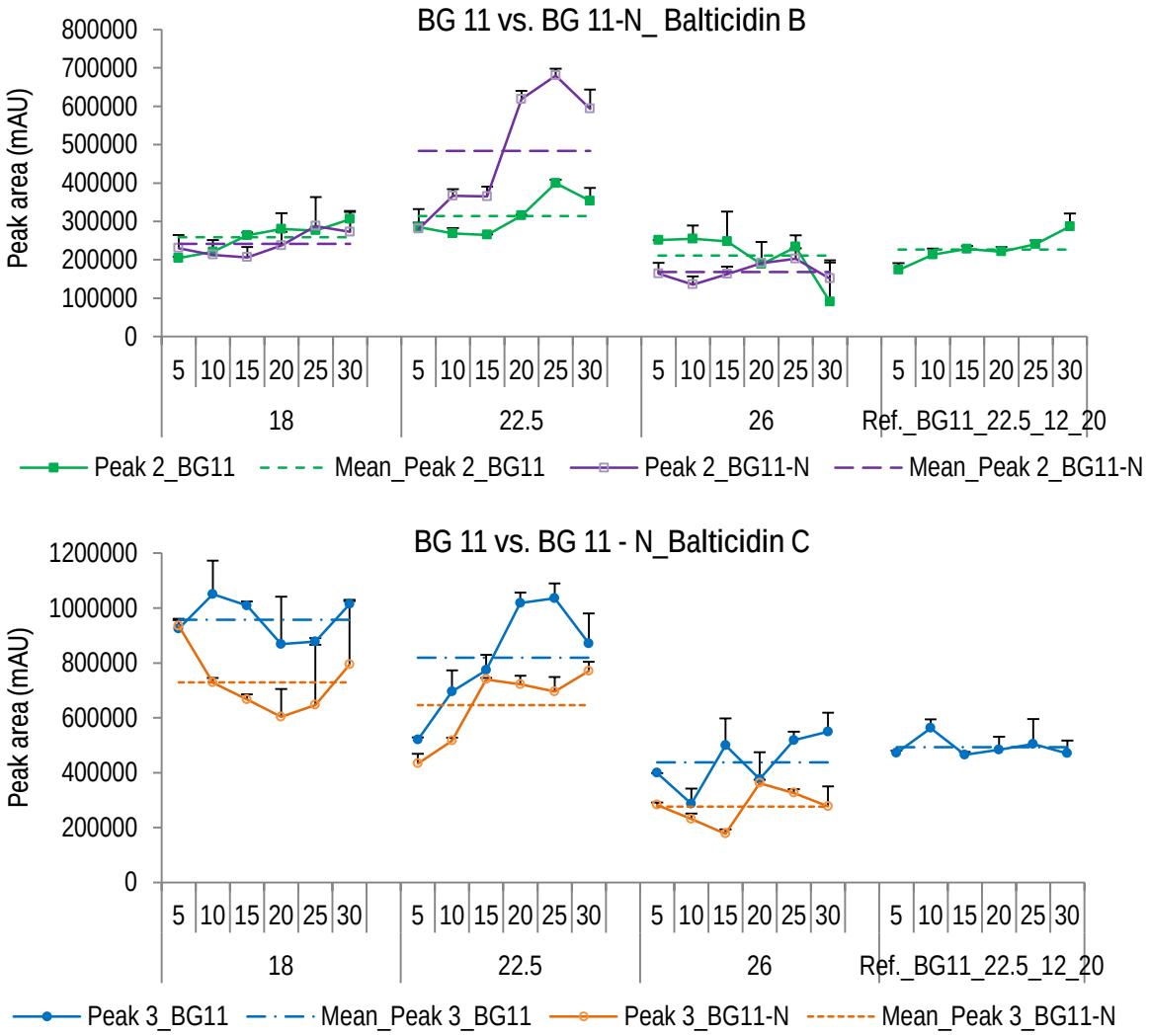


Figure III-41. Effect of nitrate on the production of balticidins A-C in BG 11 medium.
Discontinuous straight lines: mean of balticidin concentration over the cultivation time. The error bars represent the standard deviation. n = 2.

III.5.2.2. *Strain Bio 33 cultivated in MBL medium*

In MBL medium, the depletion of nitrate from MBL medium had no effect on the production of balticidins A, B, C at 22.5°C and 26°C. No significant difference in the area of peak 1, 2 and 3 between MBL medium with and without nitrate was recorded at these temperatures ($P < 0.05$).

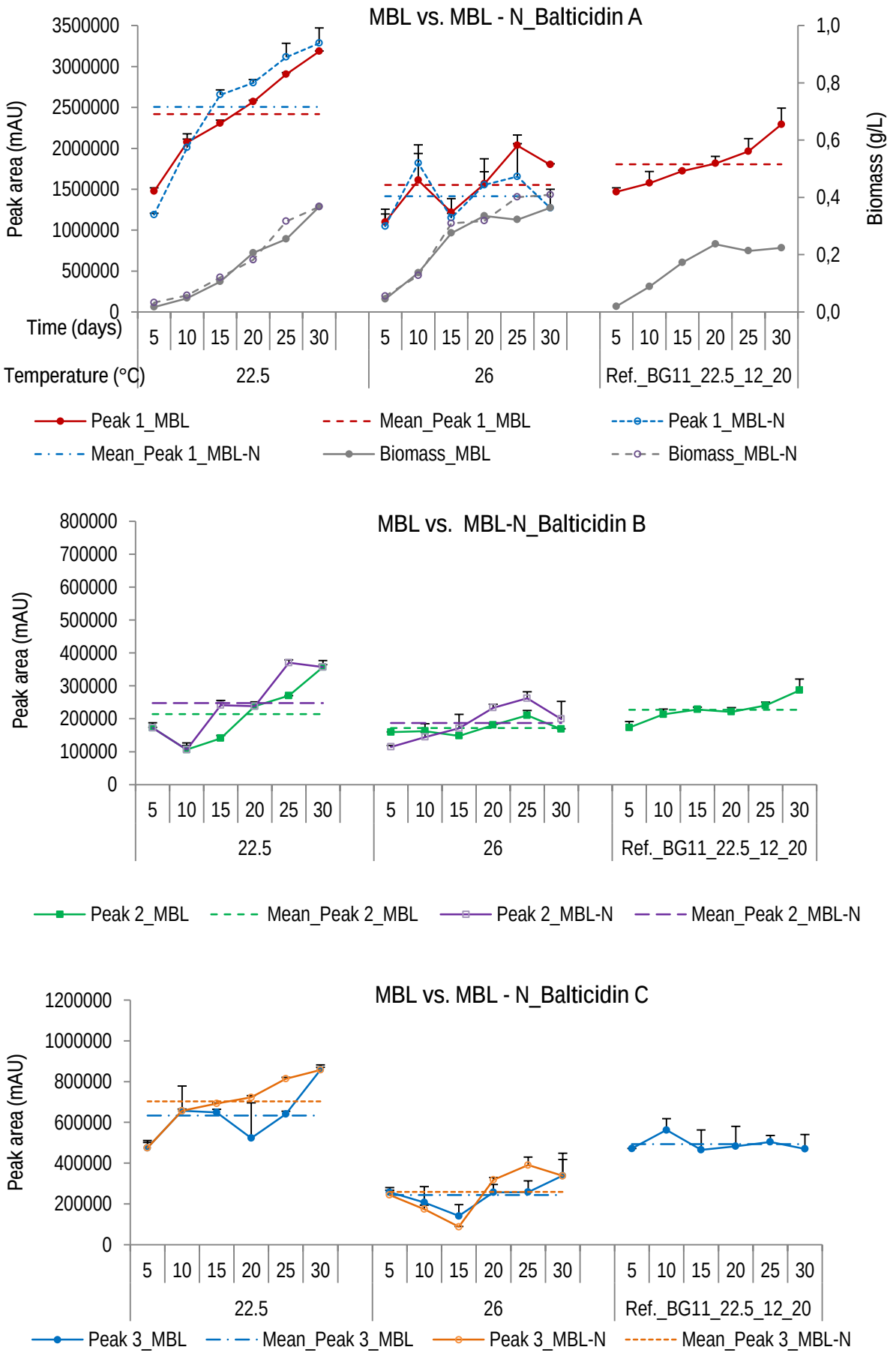


Figure III-42. Effect of nitrate on the production of balticidins A-C in MBL medium.

Discontinuous straight lines: mean of balticidin concentration over the cultivation time. The error bars represent the standard deviation. n = 2.

III.5.3. Effect of cobalt on the production of balticidins A-C

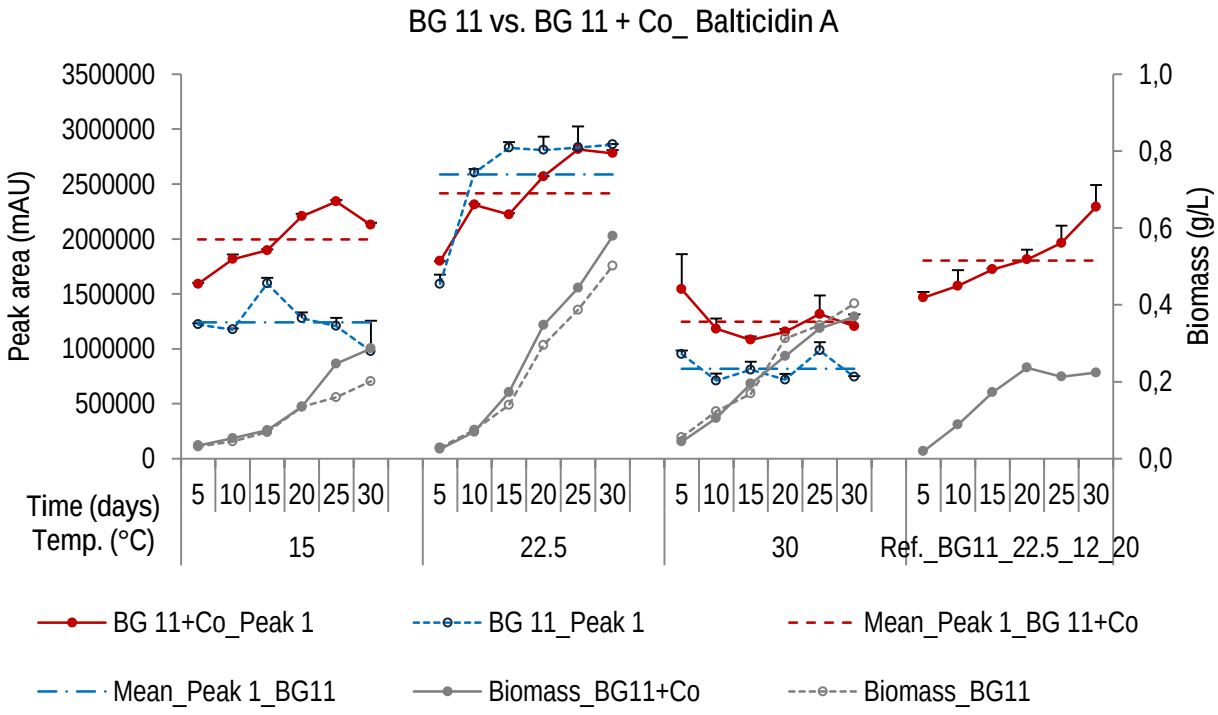
III.5.3.1. *Strain Bio 33 cultivated in BG 11 medium*

Peak 1: At 15°C and 30°C, it is obviously that cobalt had a positive effect on the production of peak 1 because a supplement of cobalt in BG 11 medium led to a significant increase of peak 1 concentration ($P < 0.01$ and $P < 0.001$, respectively). However, a supplement of cobalt to BG 11 medium at 22.5°C had no effect on the production of balticidin A; no significant difference ($P < 0.05$) in peak area was observed.

Peak 2: A supplement of cobalt to BG 11 medium had no effect on the concentration of peak 2 at 15°C (difference was not significant, $P < 0.05$) and 30°C (difference was not significant, $P < 0.05$) but decreased the concentration of peak 2 (difference was significant, $P < 0.05$) at 22.5°C.

Peak 3: The presence of cobalt in BG 11 medium at 15°C and 22.5°C increased the production of peak 3; difference in the area of peak 3 was significant ($P < 0.01$ and $P < 0.05$, respectively). At 30°C, no effect of cobalt on peak 3 was clearly observed; difference was not significant ($P < 0.05$).

In conclusion, cobalt had an obviously positive effect on the production of balticidins of Bio 33 cultivated in BG 11 medium at 15°C and 30°C. However, this effect was not clear when cobalt was added to BG 11 medium at 22.5°C – the temperature when the production of balticidins was highest.



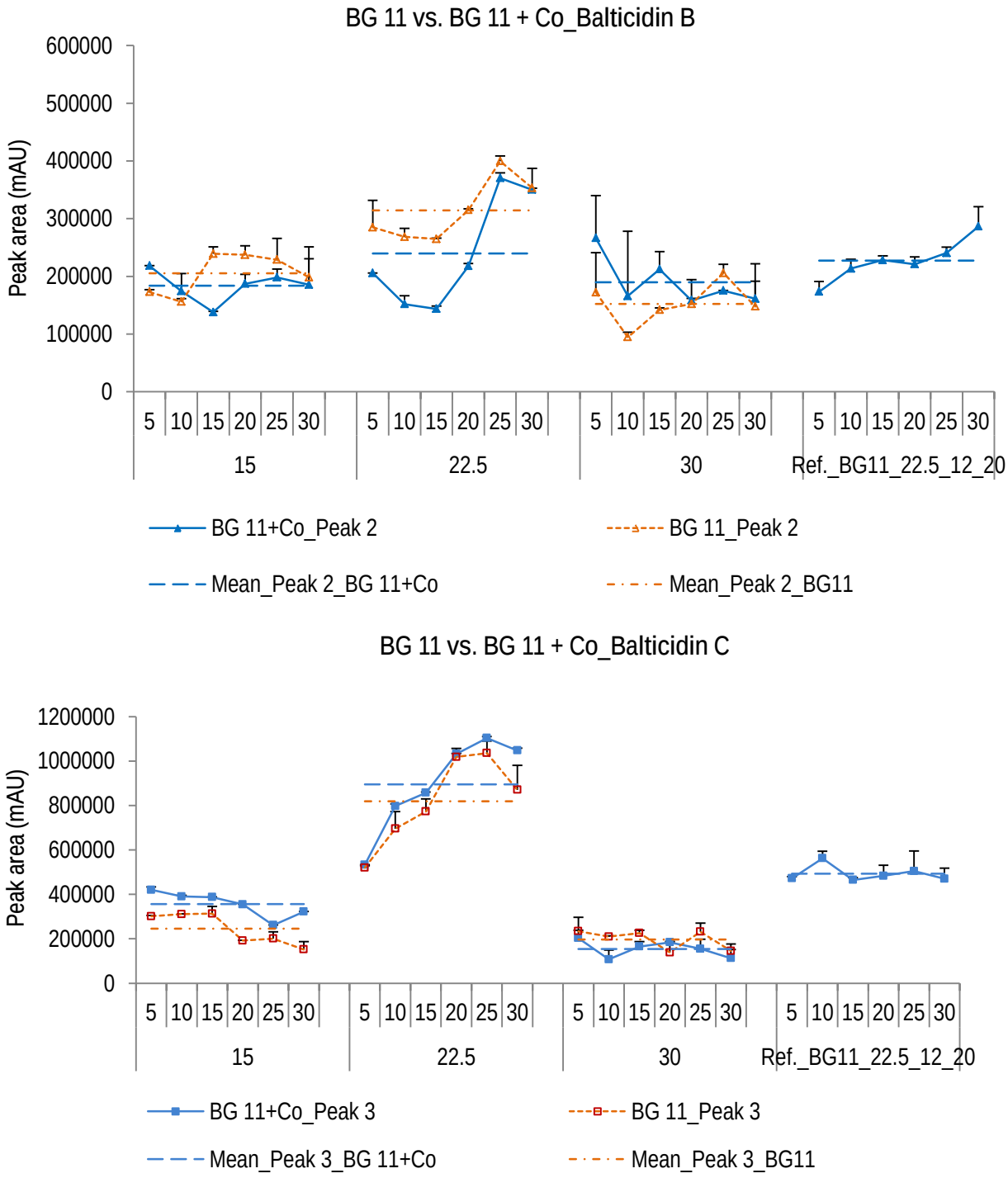
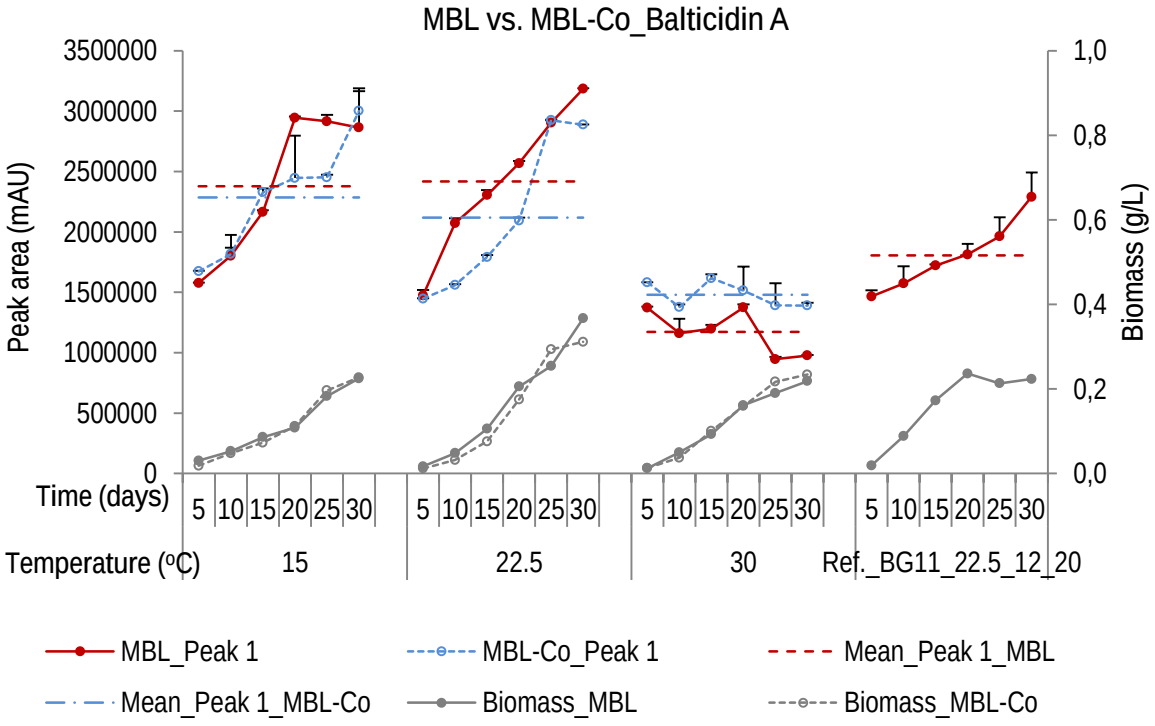
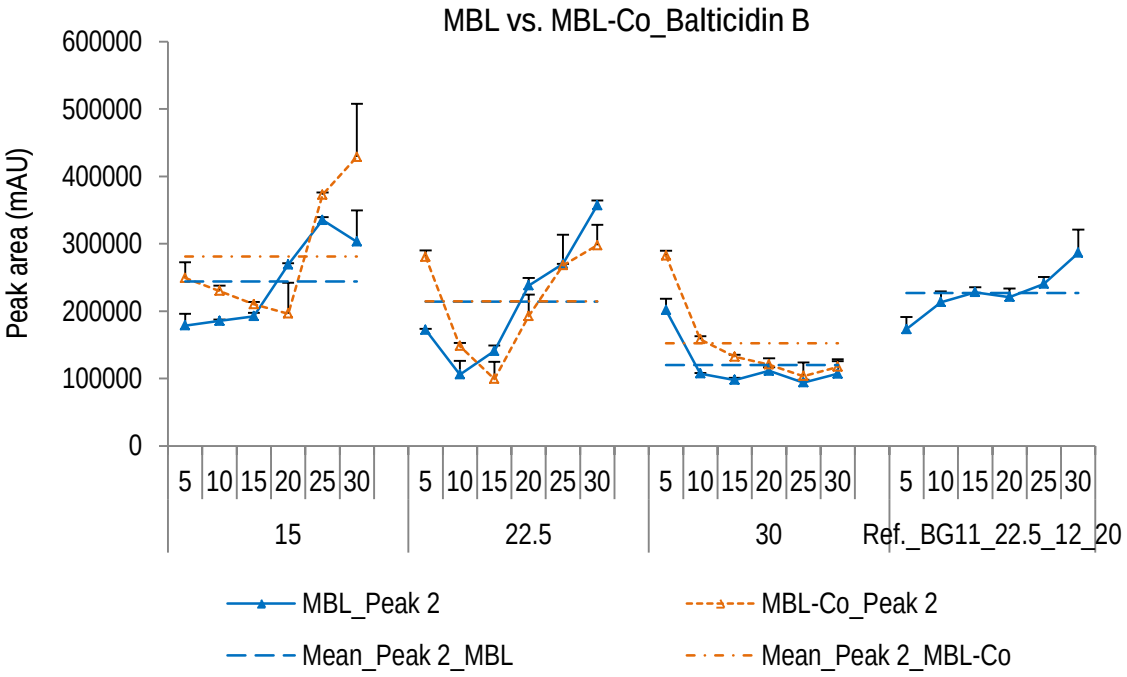


Figure III-43. Effect of cobalt on the production of balticidins A-C in BG 11 medium.
Temperature 15, 22.5 and 30°C. 24 h continuous irradiance. Light intensity 20 μmol photons m^{-2} s^{-1}. Discontinuous straight lines: mean of balticidin concentration over the cultivation time. The error bars represent the standard deviation. n = 2.

III.5.3.2. *Strain Bio 33 cultivated in MBL medium*





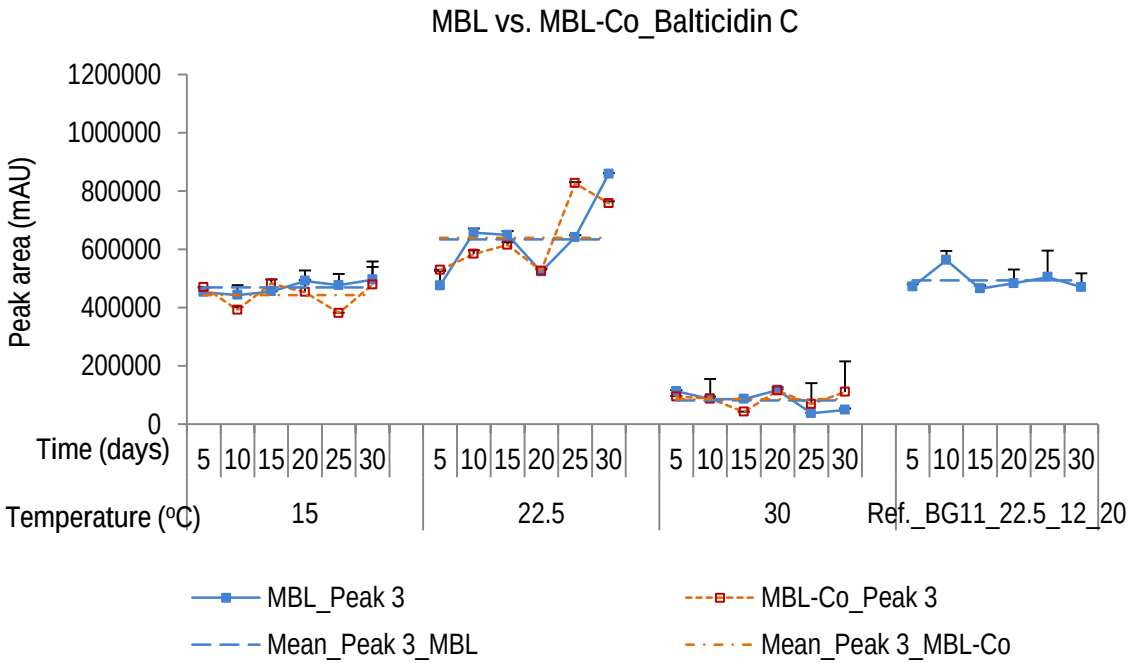


Figure III-44. Effect of cobalt on the production of balticidins A-C in MBL medium.
Temperature 15, 22.5 and 30°C. Light intensity 20 μmol photons m^{-2} s^{-1}. Irradiance of 24 h continuous light. Discontinuous straight lines: mean of balticidin concentration over the cultivation time. The error bars represent the standard deviation. n = 2.

Peak 1: The depletion of cobalt from MBL medium had no effect on the concentration of peak 1 at 15°C; difference in the area of peak 1 was not significant ($P < 0.05$). Nevertheless, the depletion of cobalt from MBL medium at 22.5°C decreased the production of peak 1 (difference was significant, $P < 0.05$) and increased it at 30°C (difference was significant, $P < 0.01$).

Peak 2: At 15°C and 22.5°C, the depletion of cobalt from MBL medium had no effect on the concentration of peak 2; no significant difference in the area of peak 2 was observed ($P < 0.05$). However, at 30°C the concentration of peak 2 of Bio 33 cultivated in MBL medium without cobalt was higher ($P < 0.05$) than in MBL medium.

Peak 3: The absence of cobalt in MBL medium did not affect the production of peak 3. No significant difference in the peak area was observed ($P < 0.05$).

In conclusion, the effect of cobalt on the production of balticidins depended on temperature. At 22.5°C, the presence of cobalt enhanced the production of balticidins in both BG 11 and MBL media. At 15°C, cobalt had no effect on the production of balticidins in MBL medium but increased it in BG 11 medium. At 30°C, cobalt had a positive effect on the production of balticidins in BG 11 medium but had a negative effect in MBL medium.

III.5.4. Effect of light intensity and time of irradiance on the production of balticidin A-C

The concentrations of peak 1, 2, 3 were studied when Bio 33 was cultivated in BG 11 and MBL medium with and without nitrate at different light intensity and time of irradiance (Figure III-45).

It is obvious that light intensity and time of irradiance had a positive effect on the production of balticidins of Bio 33. For peak 1, 2 and 3, the difference in peak area was significant in the tested media (one way ANOVA, $P < 0.0001$ (peak 1), $P < 0.01$ (peak 2), $P < 0.01$ (peak 3)). In all four media, at the same light intensity of 20 μmol photons m^{-2} s^{-1}, the production of peak 1, 2 and 3 increased when the light/dark cycle of 12 h/12 h was changed to 24 h continuous light. An experiment at 24 h continuous irradiance showed that reducing of light intensity from 20 to 10 μmol photons m^{-2} s^{-1} led to a reduced synthesis of peak 1, 2 and 3. Besides, at 24 h continuous light the concentration of peak 1, 2 and 3 increased quickly from 5th to 15th day and reached the highest concentration after 15th day. In the other hand, in case of 12 h/ 12 h light/dark rhythm the concentration of peak 1 was still increasing and had not reached the highest concentration after 30th day. In addition, a cultivation under a lower light intensity (10 μmol photons m^{-2} s^{-1}) over 24 h of irradiance in comparison to a higher light intensity (20 μmol photons m^{-2} s^{-1}) in a light/dark cycle of 12 h/12 h resulted in an enhanced synthesis of balticidins.

In conclusion, in all investigated media (BG 11 and MBL with or without nitrate), the production of balticidins was enhanced when the light intensity was increased from 10 to 20 μmol photons m^{-2} s^{-1} and the time of irradiance was increased from 12 h to 24 h light.

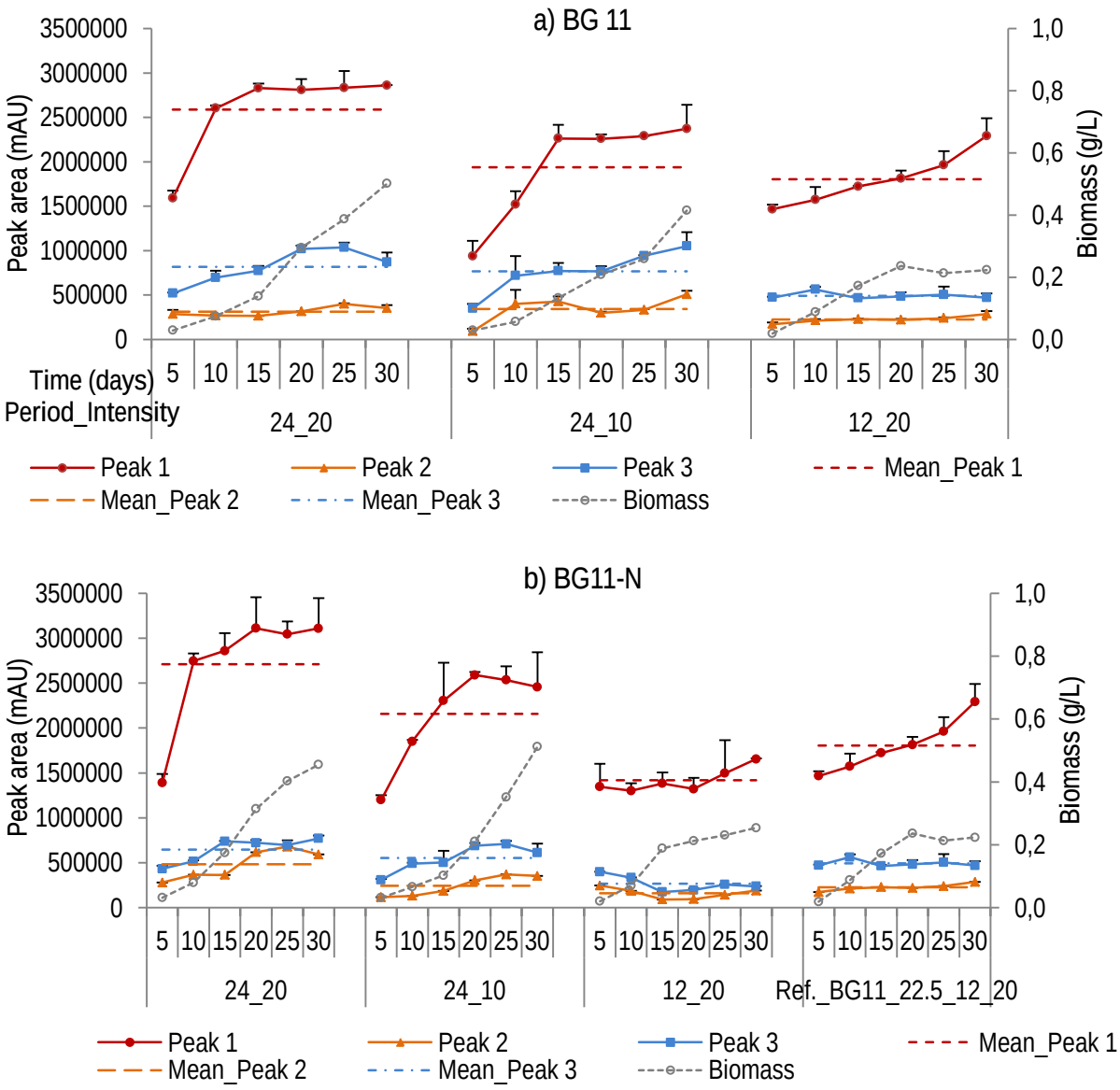


Figure III-45. Effect of light and time of irradiance on balticidins A-C of Bio 33 cultivated in BG 11 medium with and without nitrate.

a) BG 11 and b) BG 11 without $NaNO_3$. 0.5% NaCl was added to each medium. Discontinuous straight lines: mean of balticidin concentration over the cultivation time. The error bars represent the standard deviation. n = 2.

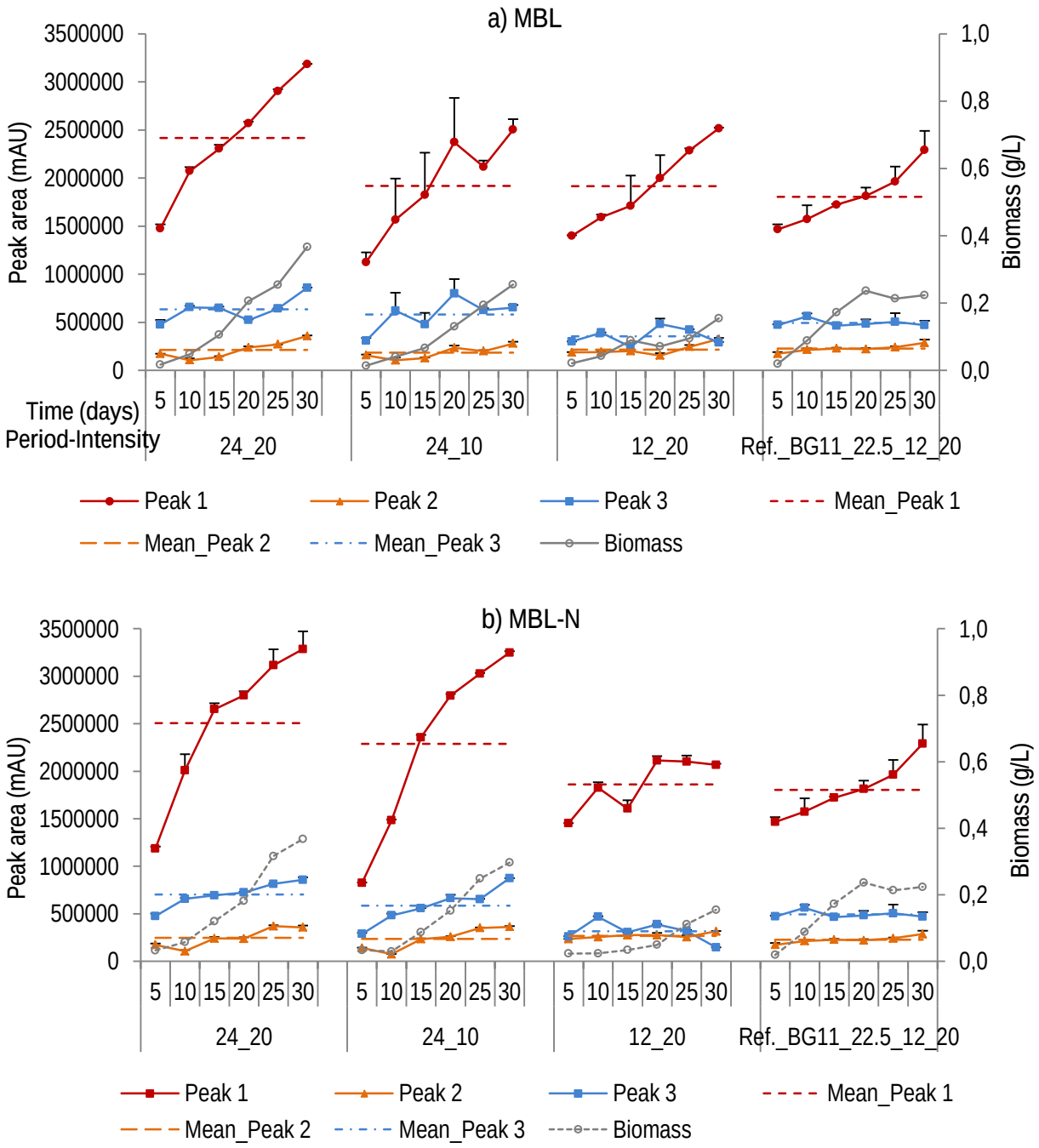


Figure III-46. Effect of light and time of irradiance on balticidins A-C of Bio 33 cultivated in MBL medium with and without nitrate.

a) MBL and b) MBL without $NaNO_3$. 0.5% NaCl was added to each medium. Discontinuous straight lines: mean of balticidin concentration over the cultivation time. The error bars represent the standard deviation. n = 2.

III.5.5. Effect of temperature on the production of balticidins A-C

III.5.5.1. *Strain Bio 33 cultivated in BG 11 medium*

Strain Bio 33 was cultivated in BG 11 medium plus 0.5% NaCl at 15, 18, 22.5, 26 and 30°C, under a 24 h continuous irradiance, at a light intensity of 20 μmol photons m^{-2} s^{-1}, and a shaking frequency of 75 rpm. The analytical HPLC of antifungal lipopeptide concentration is represented in Figure III-47.

In BG 11 medium, the difference in the area of peak 1 and 2 was extremely significant at tested temperatures (one way ANOVA, $P < 0.0001$ (peak 1), $P < 0.0001$ (peak 2)). The production of balticidin A and B was highest at 22.5°C and lowest at 30°C. The production of balticidin A and B correlated to temperature, up to an optimum at 22.5°C and then reversely correlated to the increasing temperature. A correlation analysis between cultivation time and the area of peak 1 and 2 showed that, the balticidins generally increased from the beginning of the cultivation, up to a maximum concentration at about day 15th to 20th and then decreased. Besides, the production of balticidins A and B at lowest or highest temperature reduced with cultivation time. Temperature had also an obviously effect on the production of balticidin C (difference was significant, one way ANOVA, $P < 0.0001$). The mean of balticidin C concentration over the cultivation time was highest at 18°C. However, the difference between 18 °C and 22.5 °C was not significant. Temperatures higher than 22.5°C decreased the production.

III.5.5.2. *Strain Bio 33 cultivated in MBL medium*

Strain Bio 33 was cultivated in MBL medium plus 0.5% NaCl at 15, 22.5, 26 and 30°C, under a 24 h continuous light, a light intensity of 20 μmol photons m^{-2} s^{-1}, and a shaking frequency of 75 rpm. The analytical HPLC of antifungal lipopeptide concentration is represented in Figure III-48. Difference in concentration of each balticidin A, B and C over the range of studied temperatures was significant (one way ANOVA, $P < 0.0001$ (Peak 1), $P < 0.01$ (peak 2), $P < 0.0001$ (Peak 3)). 22.5°C was the best temperature for the production of balticidin A and C, higher temperatures resulted in lower production. The lipopeptide concentration increased when the temperature increased from 15 to 22.5°C and decreased when the temperature was higher than 22.5°C. At high temperature, the production of balticidins reduced with cultivation time.

In conclusion, the optimum temperature for the production of balticidins of Bio 33 in BG 11 medium is 22.5°C; temperature higher than 22.5°C reduces the biosynthesis.

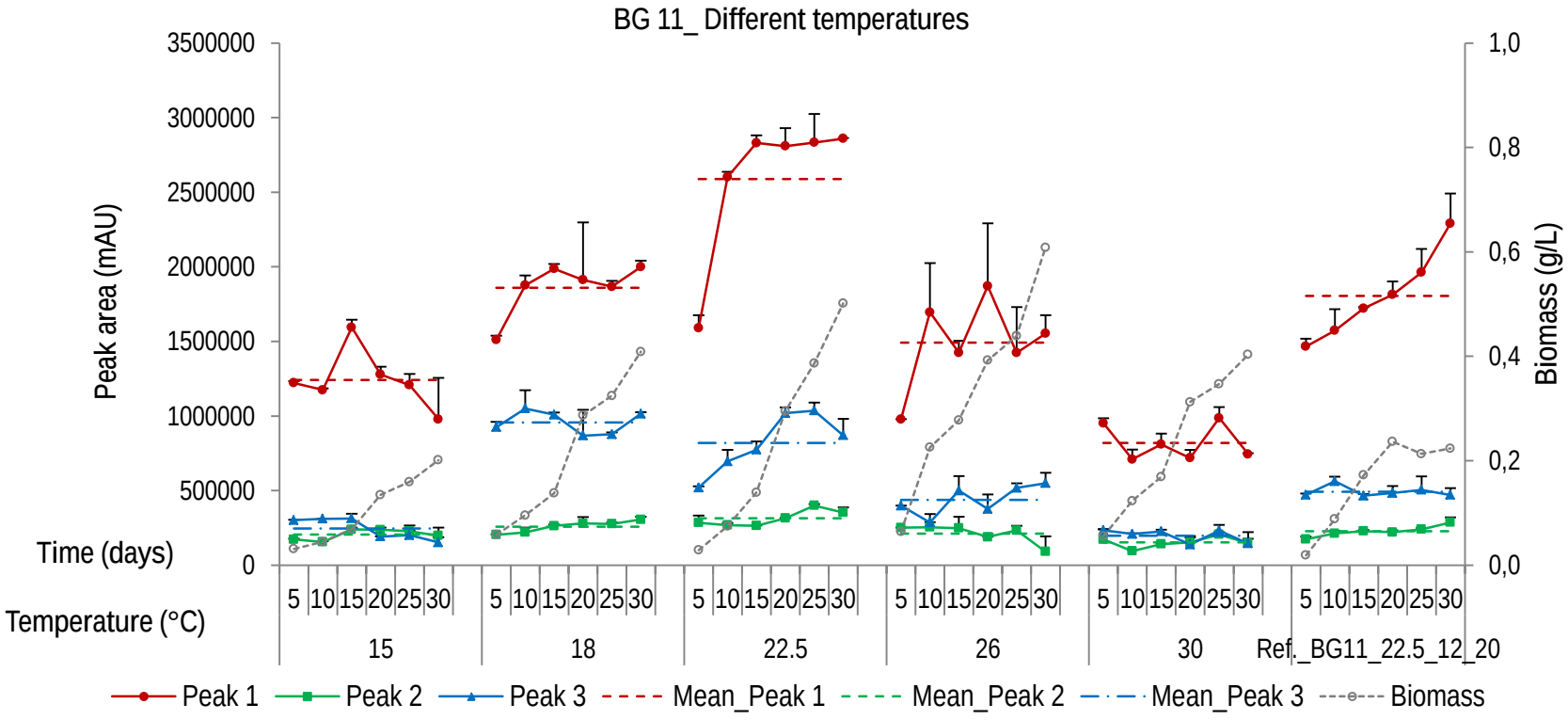


Figure III-47. Effect of temperature on balticidins A-C of Bio 33 cultivated in BG 11 medium.
Discontinuous straight lines: mean of balticidin concentration over the cultivation time. The error bars represent the standard deviation. n = 2

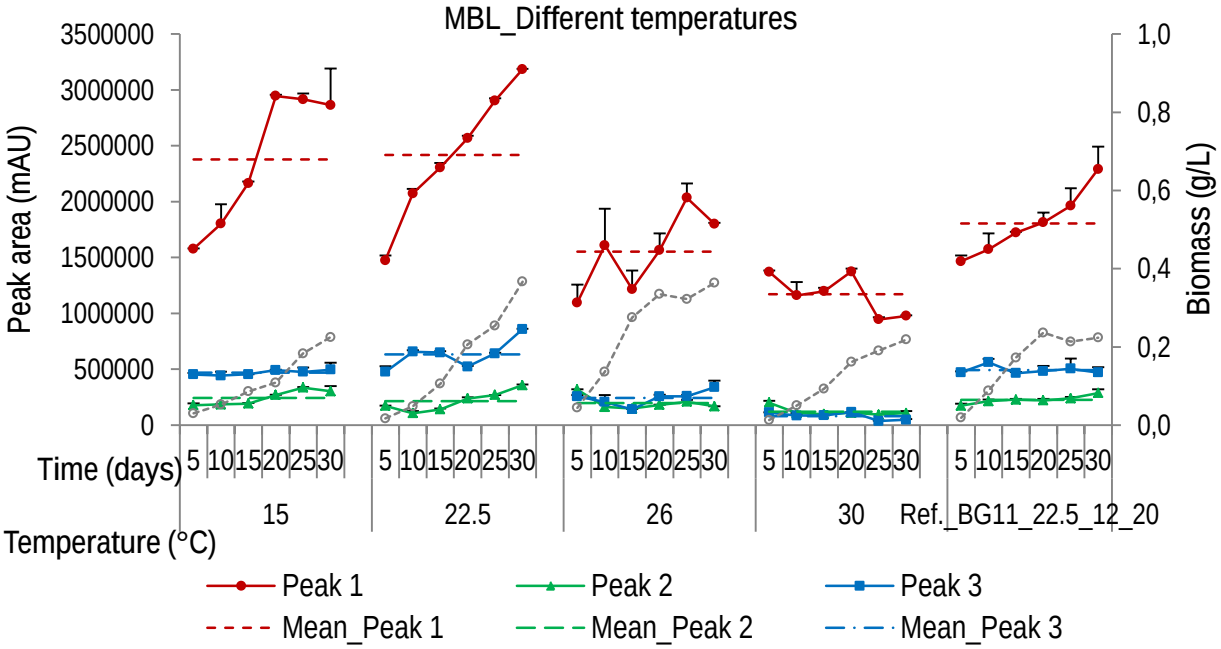


Figure III-48. Effect of temperature on balticidins A-C of Bio 33 cultivated in MBL medium.
Discontinuous straight lines: mean of balticidin concentration over the cultivation time. The error bars represent the standard deviation. n = 2.

III.5.6. Summary of culture optimization

In summary, the best conditions for the growth of Bio 33 were identified as:

- BG 11 medium,
- 24 h continuous light,
- Light intensity of 20 μmol photons m^{-2} s^{-1},
- 26°C. At this temperature, nitrate had no effect on growth of Bio 33.

Best conditions for the synthesis of balticidins A, B and C by Bio 33 were identified as:

- BG 11 medium with or without nitrate,
- 24 h continuous light,
- Light intensity of 20 μmol photons m^{-2} s^{-1},
- 22.5°C (Balticidin A); 18°C to 22.5°C (Balticidin B and C),
- Under these conditions:
    - the supplement of cobalt (0.17 μM) slightly enhanced growth but had no clear positive effect on the production of balticidins A-C.
    - the depletion of nitrate had no effect on growth and on the production of balticidin A but increased balticidin B and reduced balticidin C.

- the concentration of balticidins A, B, C reached the maximum value from 15th day to 25th day. Longer cultivation can reduce the concentration of balticidins.

III.6. Investigation of strain TVN40 and SRC

III.6.1. Growth of strain TNV40+SRC in different media

Strain TNV40+SRC was cultivated in Fernbach scale with different media including BG 11 with/without Co^{2+} and MBL with/without Co^{2+}. The dried biomass was estimated in 5 day steps.

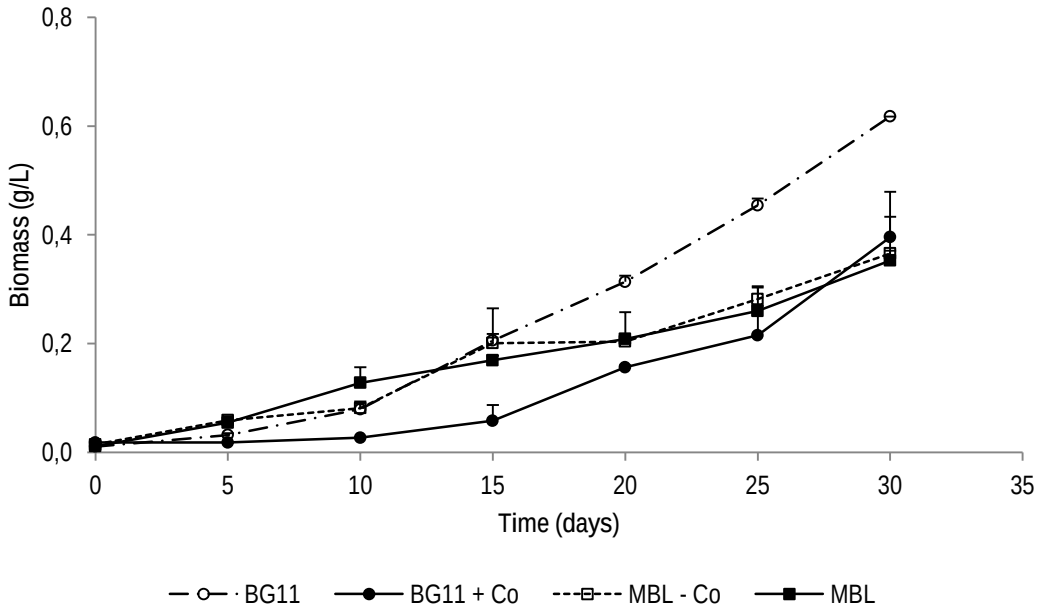


Figure III-49. Growth curves of strain TVN40+SRC in BG 11 and MBL with and without Co^{2+}.

Cultivation condition: temperature 26°C, light intensity of 10 μmol photon m^{-2} s^{-1}, shaking frequency of 100 rpm. The error bars represent the standard deviation. n = 2.

Table III-27. The specific growth rate μ and the mean doubling time G of strain TVN40+SRC grown in different media (over 10th to 30th day)

Medium	μ (log_{10} unit per day)	G (days)
BG 11	0.134	5.2
BG 11 + Co^{2+}	0.102	6.8
MBL	0.051	13.7
MBL - Co^{2+}	0.075	9.2

Based on the dry weight of biomass, BG 11 without Co^{2+} medium was the best medium for growth of strain TVN40+SRC among the four investigated media. The amount of biomass harvested at 30th day was 617.5, 395.8, 352.6, and 365.6 mg/L in BG 11, BG 11+Co, MBL, and MBL-Co media,

respectively. The presence of Co^{2+} inhibited the growth of this strain. In addition, the influence of cobalt on the growth of strain TVN40 is stronger in BG 11 medium than in MBL medium.

III.6.2. Extraction of the culture medium strain TNV40+SRC by XAD-16

For extraction of culture medium of TVN40 (BG 11 supplemented with cobalt), a volume of 6.5 L was passed through a XAD column (see II.7.2.2). After this, the column was rinsed with different solvents yielded H_2O, 80% MeOH, 100% MeOH, acetone and dichloromethane fractions as shown in Table III-28.

Table III-28. Yield of XAD fractionation from 6.5 L culture medium of strain TVN40+SRC

Fraction	m (mg/6.5 L medium; n = 2)
H_2O	108.4 ± 5.2
80% MeOH	75.7 ± 4.7
100% MeOH	8.6 ± 0.9
acetone	95.7 ± 6.2
DCM	5.3 ± 0.7

III.6.3. Antimicrobial activity of extracts from strain TVN40+SRC

EtOAc crude extract of the culture medium and all fractions from XAD column chromatography were tested against the yeast *Candida maltosa* and four bacteria as described in II.3.3. Results are represented in Table III-29 ($n \geq 3$). It was observed that the active compounds concentrated mostly in 80% MeOH, 100% MeOH and acetone fractions. H_2O fractions showed no activity while DCM fraction was active only against S .*aureus*. Largest inhibition zone (17.8 mm) was observed using 80% MeOH fraction against C. *maltosa*.

According to the antimicrobial activity and high fraction yield, the acetone and the 80% MeOH fractions were further studied to isolate the bioactive compounds.

Table III-29. Antimicrobial activity of strain TVN40+SRC

Extract/Fraction	*C.m*	*B.s*	*S.a*	*E.c*	*P.a*
EtOAc	14.0 ± 2.8	8.0 ± 0.0	8.0 ± 0.0	8.7 ± 1.2	0
H_2O	0	0	0	0	0
80% MeOH	17.8 ± 4.0	9.8 ± 2.1	8.3 ± 0.4	10.8 ± 2.0	0
100% MeOH	8.0 ± 0.0	11.8 ± 1.8	8.5 ± 0.8	10.7 ± 2.1	0
Acetone	0	8.4 ± 0.5	8.7 ± 0.6	8.5 ± 1.0	0
DCM	0	0	10.0 ± 0.0	0	0

III.6.4. Purification of the active compounds from strain TNV40+SRC by HPLC

III.6.4.1. *Purification of the acetone fraction*

Acetone fraction was purified by the method described in II.7.4.1 and the main peaks were collected. Yield of peaks is shown in Table III-30.

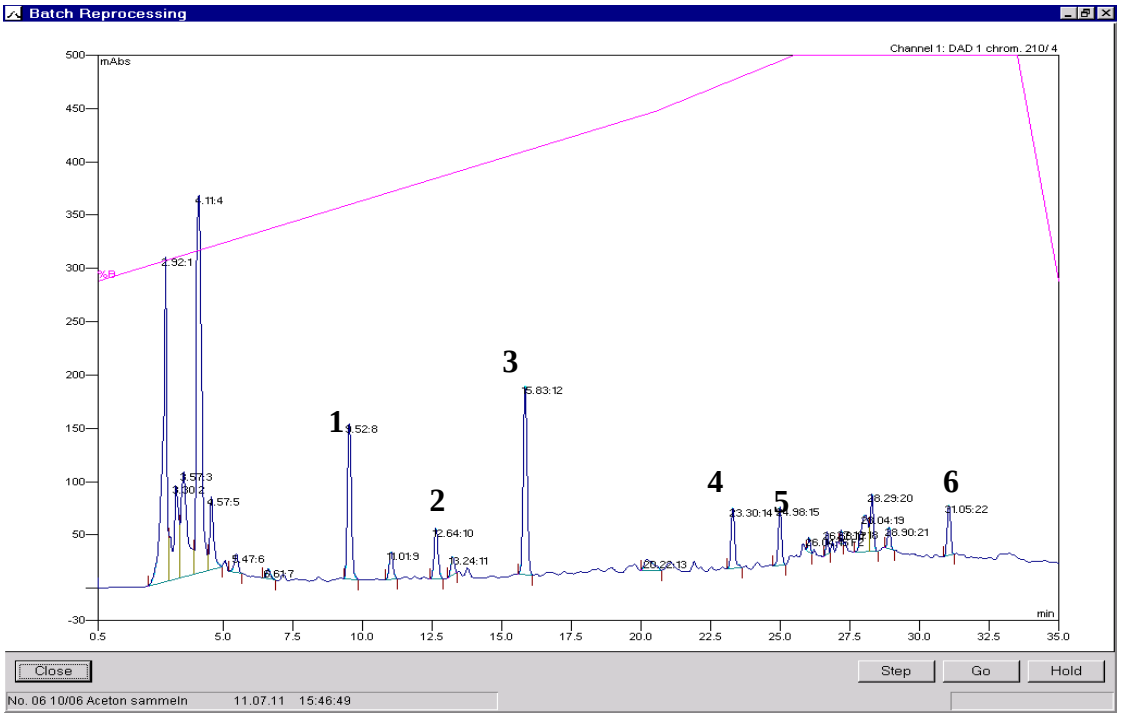


Figure III-50. Analytical HPLC chromatogram of acetone fraction isolated from the culture medium of strain TVN40+SRC.

HPLC condition: column Synergi Hydro-RP (80 Å, 250 x 4.6 mm, 4 μm). Mobile phase ACN/H_2O. Flow rate 1.0 mL min^{-1} (running program 1 (see II.7.4.1)). Injection 20 μg/20 μL/run. Detection at 238 nm.

Table III-30. Yield of isolated peaks from acetone fraction of TVN40+SRC culture medium (6.5 L)

Peak	m (mg)
1	0.0006
2	0.0004
3	0.0007
4	0.0004
5	0.0004
6	0.0005

III.6.4.2. *Purification of XAD 80% methanol fraction*

The XAD 80% MeOH fraction was purified by the method described in II.7.4.2. Peaks from the XAD 80% MeOH fraction (namely 1, 2, 3, 4, 5 see Figure III-51) were isolated by HPLC. Yield of isolated compounds (from 6.5 L medium) in sufficient amount for MS analysis were shown in Table III-31.

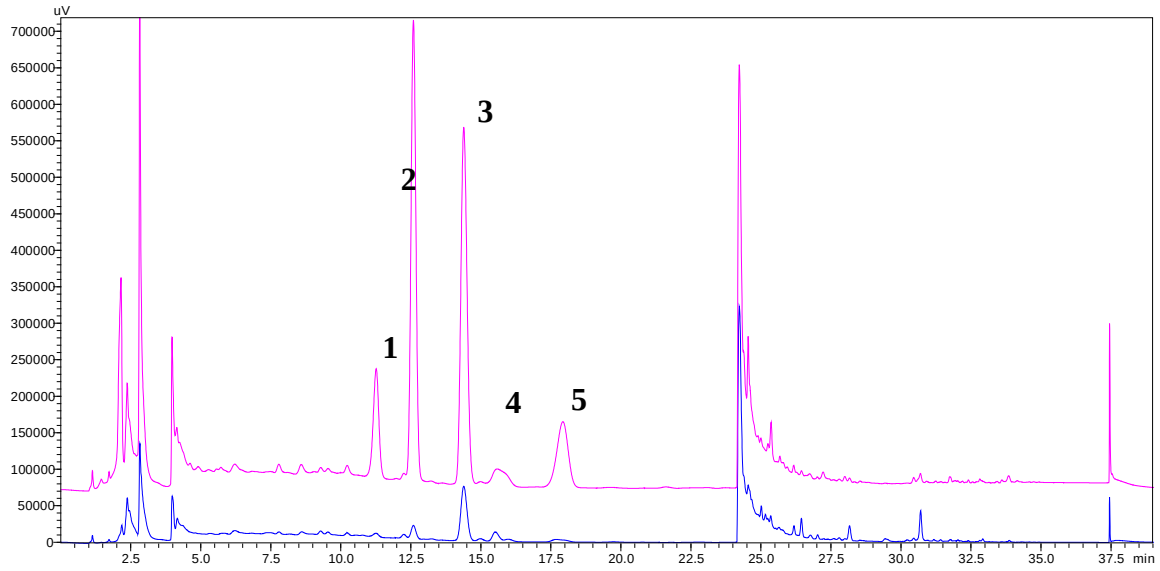


Figure III-51. Analytical HPLC chromatogram of XAD MeOH 80% fraction from the culture medium of strain TVN40+SRC.

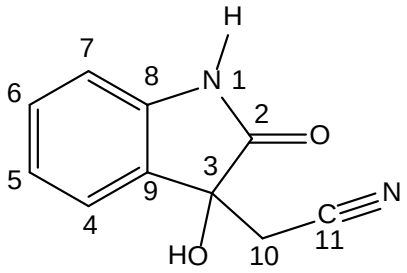
HPLC condition: column Synergi Hydro-RP (80 Å, 250 x 4.6 mm, 4 μm). Mobile phase ACN/H_2O. Flow rate 1.0 mL min^{-1} (running program 1 (see (II.7.4.2)). Injection 20 μg/20 μL/run. Detection at 210 nm (pink) and 238 nm (blue). t_{R1} = 11.261 min, t_{R2} = 12.591 min, t_{R3} = 14.389 min, t_{R4} = 17.927 min.

Table III-31. Yield of isolated peaks from XAD MeOH 80% fraction of TVN40+SRC culture medium

Peak/Compound	m (mg)
1.	1.0
2.	0.8
3.	0.4
4.	Too low for weighting
5.	0.5

III.6.5. Structural elucidation of isolated compounds of strain TVN40+SRC

The ion clusters in positive and negative ESI MS mode and the signals of ^{1}H, ^{13}C NMR of compound 3 (Table III-32) are compatible to those of a dioxindole derivative – first reported as a natural product in the work of Monde (Monde *et al.*, 1991; Suárez-Castillo *et al.*, 2006) and the following structure of compound 3 was proposed:



(3-Hydroxy-2-oxo-2,3-dihydroindol-3-yl)acetonitrile

Table III-32. NMR data of compound 3 in comparision to the reported dioxindole derivative

No.	Compound 3 (see Figure III-51) in CD_3OD			Compound 4 in (Suárez-Castillo *et al.*, 2006) in aceton-d_6	
	δ ^{13}C [ppm]	δ ^{1}H [ppm] m (*J* [Hz])	HMBC to C	δ ^{13}C [ppm]	δ ^{1}H [ppm] m (*J* [Hz])
2	179.3	---		178.1	---
3	73.9	---		74.0	---
4	125.4	7.546 ddd (7.5/1.3/0.6)	3, 6, 8	125.9	7.62 d (7.3)
5	124.0	7.099 ddd (7.6/7.5/0.9)	7, 9	124.0	7.13 t (7.7)
6	131.6	7.322 ddd (7.8/7.6/1.3)	4, 8	131.8	7.36 t (7.7)
7	111.6	6.930 ddd (7.8/0.9/0.6)	5, 9	111.9	7.02 d (8.1)
8	142.7	---		143.1	---
9	130.7	---		130.8	---
10	27.2	3.068 d (16.6) / 2.874 d (16.6)	2, 3, 9, 11	27.8	3.19 d (16.6) / 2.98 d (16.6)
11	117.3	---		117.7	---

s: singlet, d: doublets, t: triplets and m: multiplets

Masses of $[M+Na]^+$ and $[M-H]^-$ of other isolated compounds were estimated by HPLC-ESIMS and are listed in Table III-33 and Appendix VII. The identification of the other isolated compounds from strain TVN40 is still in process.

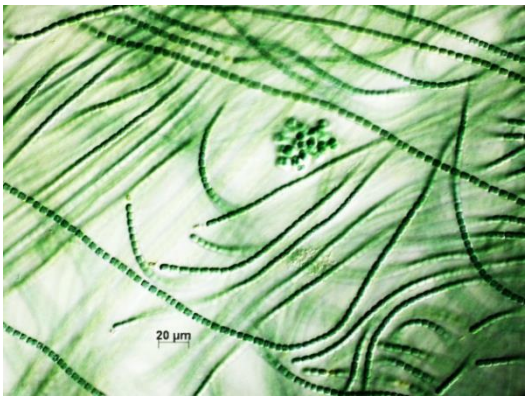
Table III-33. Mass spectrometric data of three compounds 1, 2, and 5

Compound	$[M+Na]^+$ (*m/z*)	$[M-H]^-$ (*m/z*)
1	601.262	577.268
2	625.262	601.266
5	639.277	615.283

III.6.6. Purification of the TVN40 culture

To identify the origin of the antimicrobial activity of the extracts of TVN40, we isolated the filamentous TVN40 and the strange round cells (SRC) by methods described in II.9. Both TVN40 and SRC were cultivated in BG 11 medium and BG 11 supplemented with Co^{2+}. The culture medium and biomass were extracted and tested for antimicrobial activity.

a)



b)

c)

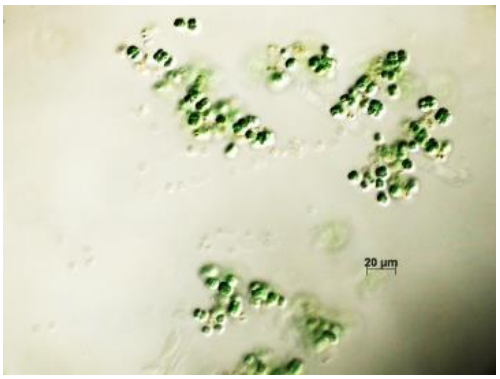


Figure III-52. Isolation of fiamentous and unicellular cyanobacteria.

a) Sample containing TVN40 and SRC before isolation (magnification 400x); b) Isolated filamentous of TVN40 (magnification 400x); c) Isolated unicellular SRCs (magnification 400x).

III.6.7. Comparison of extraction of filamentous TNV40, SRC and strain TVN40+SRC

Filamentous TVN40 and SRC were cultivated in Fernbach flasks (see II.1.2.4) with BG 11 medium and BG 11 supplemented with Co^{2+}. Strain TVN40+SRC was cultivated in large scale (see II.1.2.5). The dried biomass was estimated (Table III-34) and extracted with *n*-hexane, MeOH and H_2O (see II.2.2.1).

A volume of 3 L of each culture was extracted with Amberlite™ XAD-16 (see II.7.2.2). In parallel a volume of 3 L of each medium was extracted with EtOAc (see II.2.2.2). The yields of extraction and XAD fractions are represented in Table III-35.

A higher yield of MeOH and MeOH/H_2O extracts was recorded in comparison to *n*-hexane and water extracts. In general, the percentage of MeOH extract ranged from 10.6 to 17.8%, of MeOH/H_2O extract from 5.9 to 16.1%, and of *n*-hexane extract from 0.6 to 2.1%. The percentage of MeOH extract of isolated TVN40 or isolated SRC was lower than that of the biomass harvested from the cocultivation of both strains.

Table III-34. Amount of biomass from strain TVN40 and SRC cultivated in different media

	Medium	Biomass (g/L)	Cultivation scale
TVN40+SRC_BG11+Co	BG 11 + Co^{2+}	0.42	big column (35 L medium), aeration, pH 8.5, 26°C
TVN40+SRC_BG11	BG 11	0.46	big column (35 L medium), aeration, pH 8.5, 26°C
SRC_BG11+Co	BG 11 + Co^{2+}	0.60	big column (35 L medium), aeration, pH 8.5, 26°C
TVN40 _BG11+Co	BG 11 + Co^{2+}	0.23±0.02	10 Fernbach flasks (7 L medium), 16-20°C
TVN40 _BG11	BG 11	0.18±0.03	10 Fernbach flasks (7 L medium), 16-20°C

Table III-35. The yield of extracts of strain TVN40 and SRC cultivated in different media

	Extracts				
Medium	*n*-hexane (%)	MeOH (%)	MeOH/H_2O (1:1) (%)	H_2O (%)	EtOAc (mg/3L)
TVN40+SRC_BG11+Co	0.73	16.81	15.22	1.97	8.09
TVN40+SRC_BG11	0.59	17.76	10.07	3.38	8.35
TVN40 _BG11+Co	0.55	13.23	16.09	1.91	5.1
TVN40 _BG11	2.13	13.40	12.41	1.06	4.7
SRC_BG11+Co	2.04	10.61	5.89	2.70	4.6

XAD fractions of culture media (mg/3L medium)

Medium	H_2O wash	MeOH 80%	MeOH 100%	Acetone	DCM
TVN40+SRC_BG11+Co	20.5	75.7	8.6	95.7	5.3
TVN40+SRC_BG11	-*	-	-	-	-
TVN40 _BG11+Co	71.0	7.7	11.1	82.1	11.2
TVN40 _BG11	97.9	7.4	8.2	65.0	7.0
SRC_BG11+Co	54.85	7.1	4.0	70.3	0.4

*Experiment was not done with strain TVN40+SRC cultivated in BG 11 medium (named TVN40+SRC_BG11).

III.6.8. Comparison of antimicrobial activity of the isolated TVN40, SRC and strain TVN40+SRC

The two isolated cyanobacteria (TVN40 and SRC, see III.6.6) were cultivated in media BG 11 with and without Co^{2+} and the antimicrobial activity of the extra- and intracellular extracts in agar diffusion test were compared to the results of strain TVN40+SRC. Only the *n*-hexane extracts of the TVN40 cocultivated with the SRCs exhibited activity against both *B. subtilis* and *S. aureus*. When the SRCs

were removed from the culture and a pure culture of the filamentous TVN40 existed, the *n*-hexane extracts were only active against *S. aureus*. Otherwise, the *n*-hexane extracts of the pure SRCs also exhibited a low but visible inhibition of the growth of *S. aureus* only. No antimicrobial activity of MeOH and water extracts of all biomasses was observed (see Table III-36). The same result was described by Le (Le, 2010) and Heinek (Heinek, 2011).

Table III-36. Antimicrobial activity of the biomass extracts from strain TVN40+SRS, TVN40 and SRS cultivated in different media

Strain_Medium	Extract	Inhibition zone (mm)				
		C. maltosa	*P. aeruginosa*	*E. coli*	*S. aureus*	*B. subtilis*
TVN40+SRC_BG11+Co	*n*-hexane	0	0	0	10 ± 0	8.0 ± 1.7
	MeOH	0	0	0	0	0
	H_2O	0	0	0	0	0
TVN40+SRC_BG11	*n*-hexane	0	0	0	9.75 ± 1.1	10 ± 0
	MeOH	0	0	0	0	0
	H_2O	0	0	0	0	0
SRC_BG11+Co	*n*-hexane	0	0	0	8.3 ± 1.5	0
	MeOH	0	0	0	0	0
	H_2O	0	0	0	0	0
SRC_BG11	*n*-hexane	0	0	0	8.0 ± 0	0
	MeOH	0	0	0	0	0
	H_2O	0	0	0	0	0
TVN40 _BG11+Co	*n*-hexane	0	0	0	12 ± 0	0
	MeOH	0	0	0	0	0
	H_2O	0	0	0	0	0
TVN40 _BG11	*n*-hexane	0	0	0	9.5 ± 0	0
	MeOH	0	0	0	0	0
	H_2O	0	0	0	0	0

n = 3. 2 mg/6 mm paper disc. "0" means no activity found.

The antimicrobial activity of the EtOAc extracts and the XAD fractions of the culture medium against *C. maltosa*, *B. subtilis*, *S. aureus* and *E. coli* are represented in Figure III-53 to Figure III-57.

The EtOAc extracts of the TVN40 growing together with SRC showed activity against *C. maltosa*, *B. subtilis*, *S. aureus* and *E. coli*. Tests on the EtOAc extract of the pure TVN40 showed the same results but with higher activity. However, the EtOAc extract of the pure SRC exhibited activity only against the three bacteria; no activity against *C. maltosa* was observed. The H_2O fractions of all strains showed no inhibition zone.

The activity against *C. maltosa* was observed in the 80% MeOH and 100% MeOH fractions of TVN40 and TVN40+SRC but not in acetone and dichloromethane fractions. Besides, this activity of the mixed culture TVN40+SRC was higher than that of the pure TVN40.

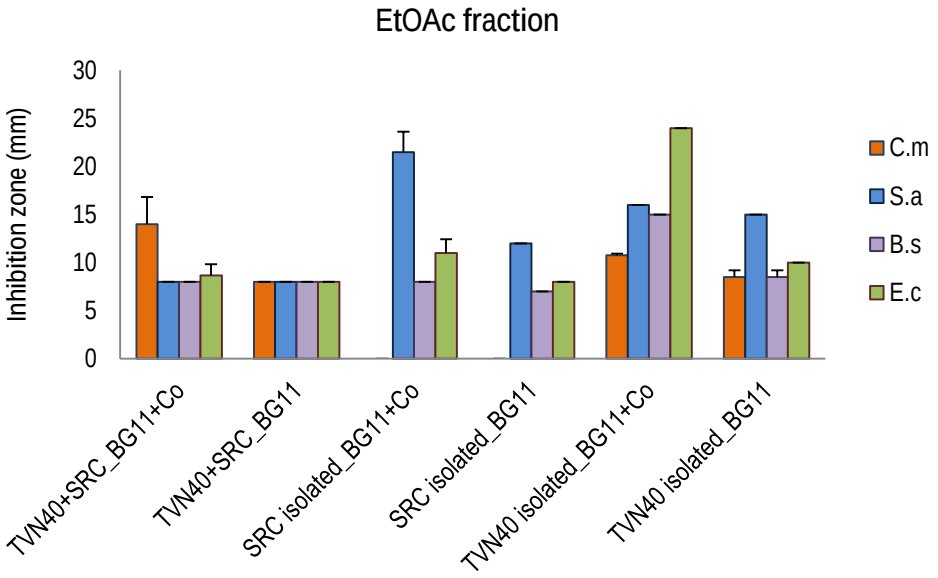


Figure III-53. Antimicrobial activity of the EtOAc extract of culture medium of strain TVN40+SRC, TVN40 and SRC.

Trains were cultivated in different culture media. Test concentration 1 mg/6 mm paper disc. n = 3.

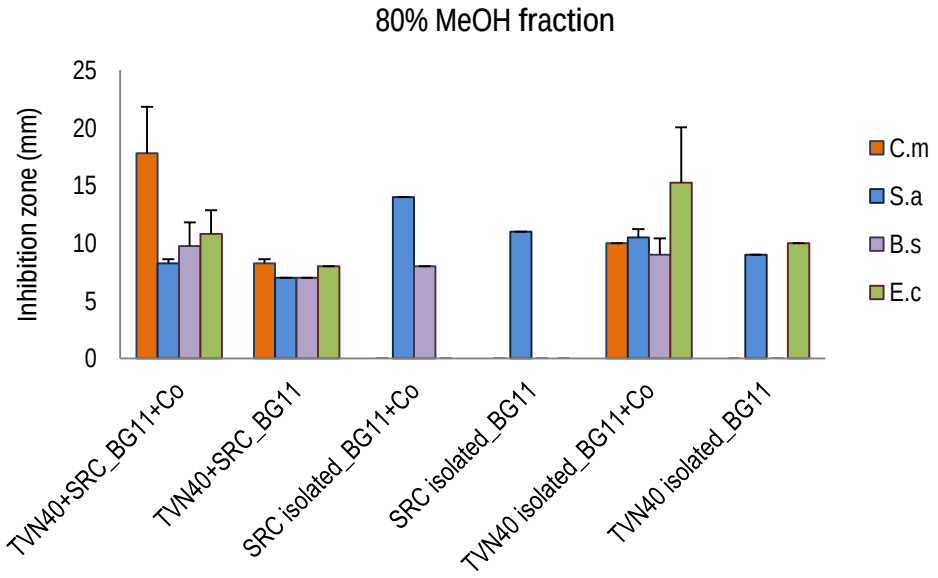


Figure III-54. Antimicrobial activity of the 80% MeOH fraction of culture medium of strain TVN40+SRC, TVN40 and SRC.

Trains were cultivated in different culture media. The fraction was separated by XAD column. Test concentration 1 mg/6 mm paper disc. n = 3.

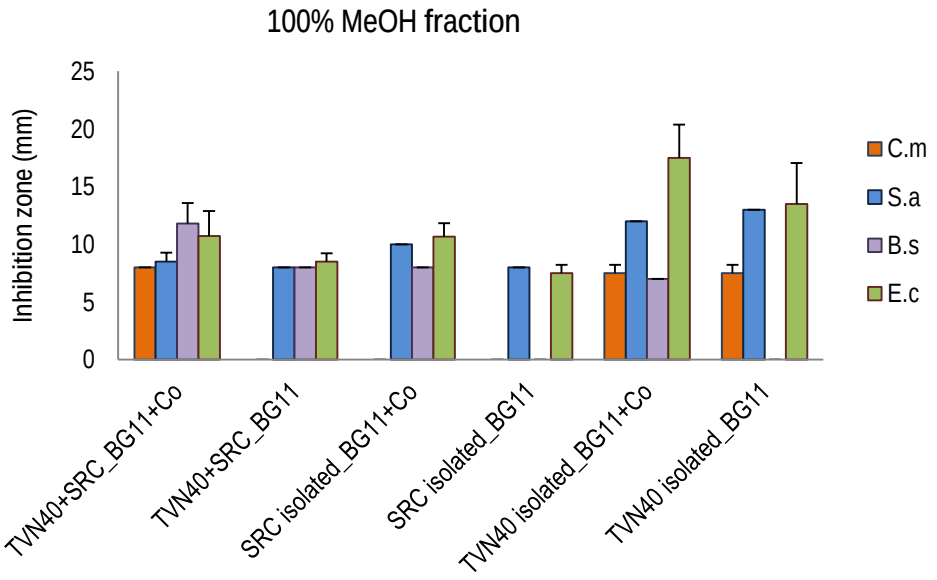


Figure III-55. Antimicrobial activity of the 100% MeOH fraction of culture medium of strain TVN40+SRC, TVN40 and SRC.

Trains were cultivated in different culture media The fraction was separated by XAD column. Test concentration 1 mg/6 mm paper disc. n = 3.

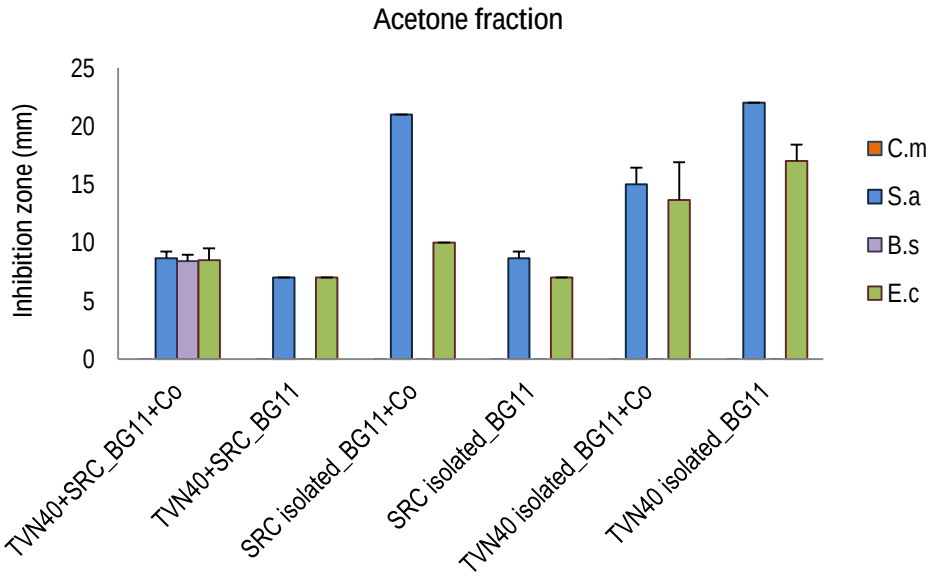


Figure III-56. Antimicrobial activity of the acetone fraction of culture medium of strain TVN40+SRC, TVN40 and SRC.

Trains were cultivated in different culture media The fraction was separated by XAD column. Test concentration 1 mg/6 mm paper disc. n = 3.

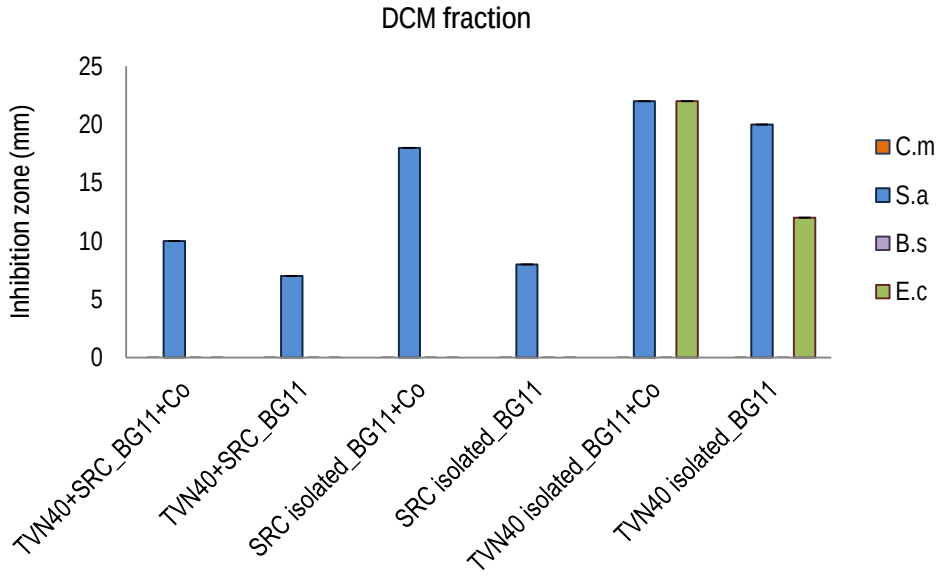


Figure III-57. Antimicrobial activity of the dichloromethane fraction of culture medium of strain TVN40+SRC, TVN40 and SRC.

Trains were cultivated in different culture media. The fraction was separated by XAD column. Test concentration 1 mg/6 mm paper disc. n = 3.

The activity against *B. subtilis*, *S. aureus* and *E. coli* was found in all fractions of all strains. Nevertheless, the comparison of inhibition zones showed that the activity of the pure TVN40 and SRC was stronger than that of the mixed culture of TVN40+SRC. The EtOAc extract and XAD fractions of the mixed culture of TVN40+SRC as well as of the pure TVN40 showed activity against *C. maltosa*, but that of the pure SRC showed no antifungal activity. It is unambiguously that TVN40 is the strain, which was responsible for the antifungal activity of the mixed culture of TVN40 and SRC. The agardiffusion assay also showed that the presence of cobalt in the culture medium increased the inhibition zone of the EtOAc extracts and XAD-16 fractions.

In conclusion, according to the agardiffusion assay, both TVN40 and SRC culture media were responsible for the antibacterial activity against *B. subtilis*, *S. aureus* and *E. coli* because (i) the medium extracts of the two pure strains and the mixed culture were active, (ii) the activity was present in all of the medium extracts. However, only the extract of the culture medium of TVN40 was active against *C. maltosa*. The supplement of cobalt enhanced the antimicrobial activity of the culture medium. Pure strains showed higher activity in comparison to the mixed culture of TVN40 and SRC.

III.6.9. Isolation, cultivation, extraction and tesing of isolated bacteria from SRC

Type and number of bacterial colonies grew on each type of agar plate were compared. As a result, 4 bacteria were isolated and named as: 1) white, large; 2) pale yellow, small; 3) yellow; 4) orange, Malt. The bacterium number 4) was isolated from a Malt agar plate. Besides, TYG medium was considered

as the best medium for cultivation of the isolated bacteria. Yield of extraction and agar diffusion assay are summarized in the following table:

Extract		Yield g/L medium	Bacteria				
			S.a	*B.s*	*E.c*	*P.a*	*C.m*
EtOAc extract	[a]BA_culture medium	0.0393	9	12	9	-	10
	TYG medium	0.0226	-	-	7, uc	-	9, uc
Bacterial biomass	[b]BA biomass	1.0484					
	n-hexane extract	0.07%					-
	MeOH extract	39.5%	-	-	-	-	-
	H_2O extract	11.2%	-	-	-	-	-

[a]Bacterial culture medium. [b]Bacterial biomass. Concentration of extract 2 mg/paper disc.

The EtOAc extract of bacterial culture medium showed a weak activity against *S. aureus*, *B. subtilis*, *E. coli* and *C. maltosa*. Besides, the EtOAc extract of the control TYG medium also displayed a weak activity against *E. coli* and *C. maltosa*.

III.6.10. ESI MS analysis for the presence of isolated compounds

The presence of *m/z* values according to $[M+Na]^+$ and $[M-H]^-$ of these five compounds - fluorensadiol, the dioxindole derivative and compound 1, 2, and 5 (see Table III-33) - was checked and the results are presented in Table III-37.

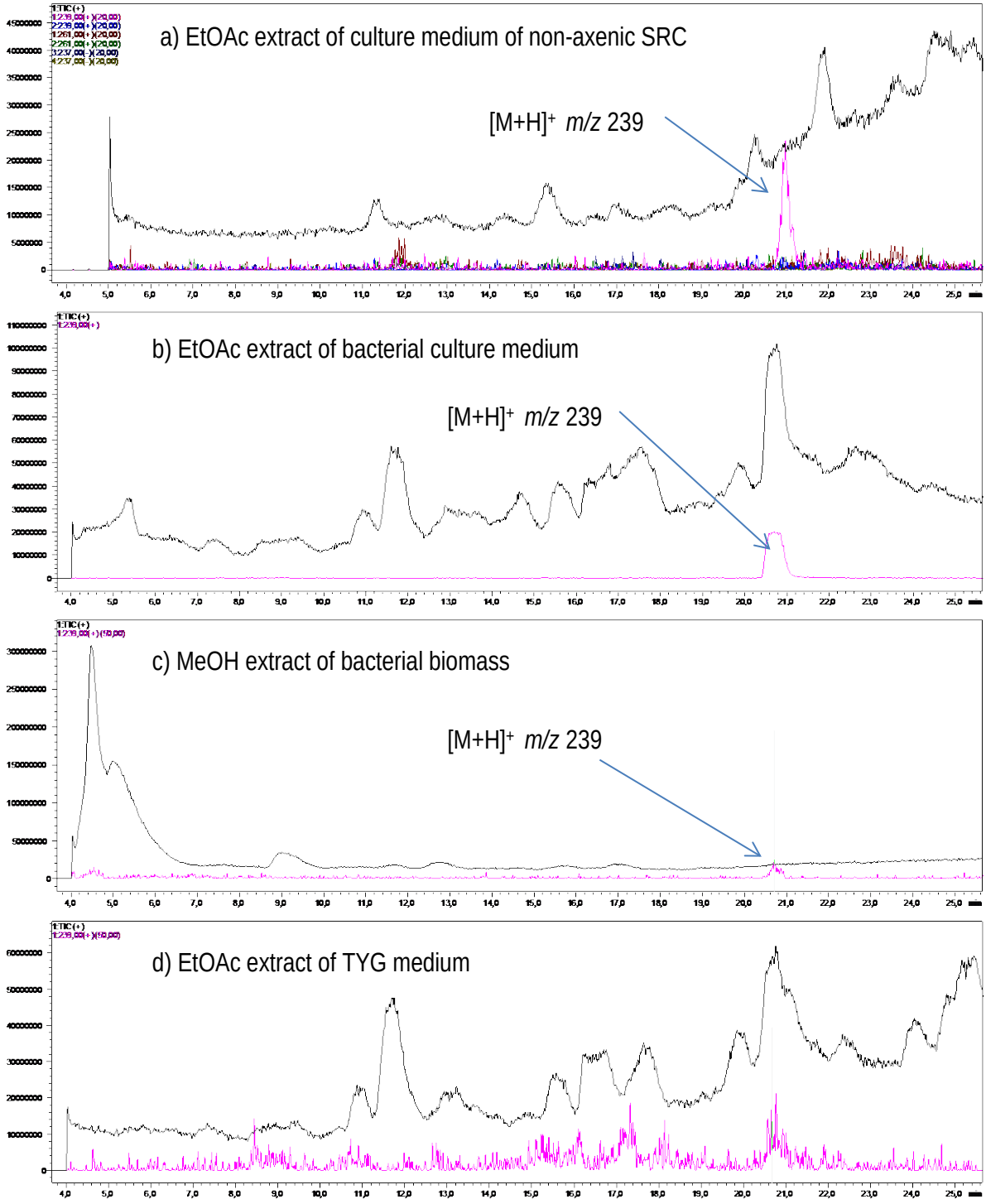
Table III-37. Presence of *m/z* peaks according to $[M+Na]^+$ and $[M-H]^-$ in HPLC/MS

Sample / Compound	Flourensadiol	Dioxindole derivative	Compound 1	Compound 2	Compound 5
XAD 80% MeOH fraction of strain TVN40+SRC	-	+	+	+	+
All other XAD fractions of strain TVN40+SRC	-	na	na	na	na
EtOAc extract of strain TVN40+SRC	-	na	na	na	na
EtOAc extract of bacterial culture medium	+	+	-	-	-
MeOH extract of bacterial biomass	+	-	-	-	-
EtOAc extract of TYG medium	-	+	-	-	-
EtOAc extract of culture medium (BG 11+Co) of SRCs	+	+	-	-	-
EtOAc extract of culture medium (BG 11+Co) of strain TVN40	+	+	-	-	-
EtOAc extract of culture medium (BG 11-Co) of strain TVN40	+	+	-	-	-

"+": present; "-": absent; "na": not analyzed

III.6.10.1. *ESI MS analysis for the presence of flourensadiol*

As a result of scan mode, a peak with *m/z* 239 according to $[M+H]^+$ of flourensadiol (M = 238) in positive mode was found (see Figure III-58). TYG medium showed no peak with *m/z* 239. The intensity of peak *m/z* 239 was highest in the culture medium extract of the bacteria isolated from SRC (see Index VIII for the mass spectrum).



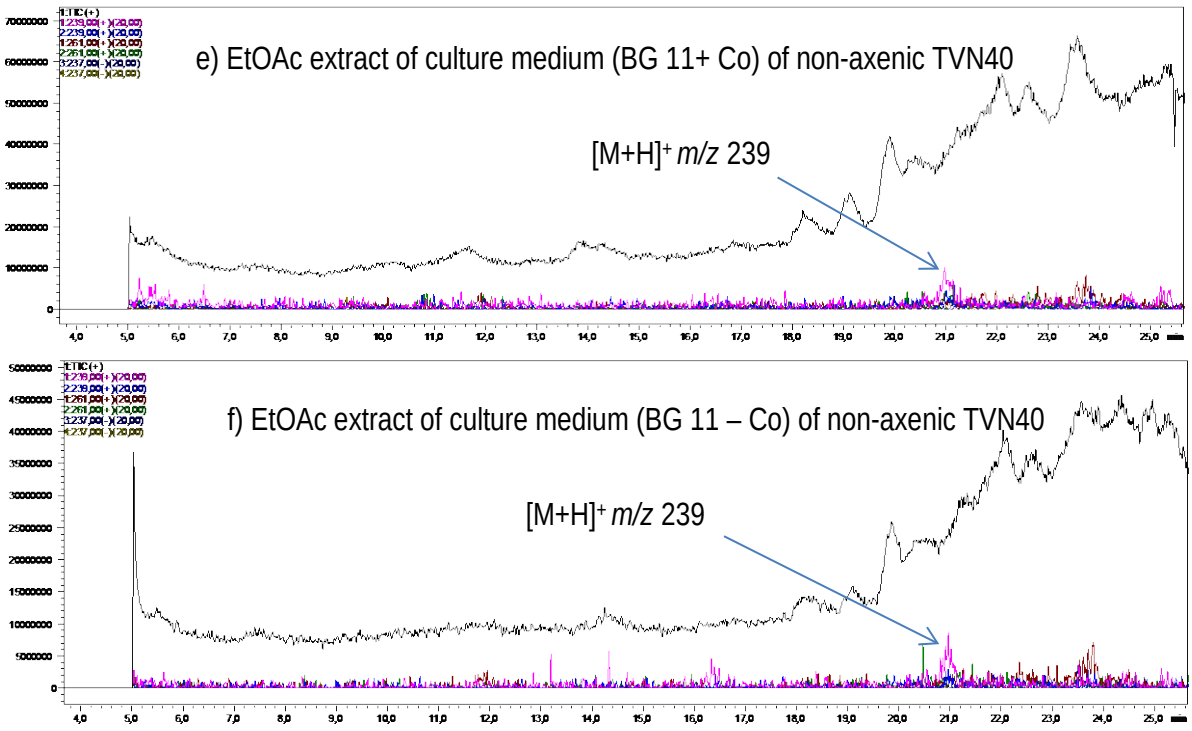
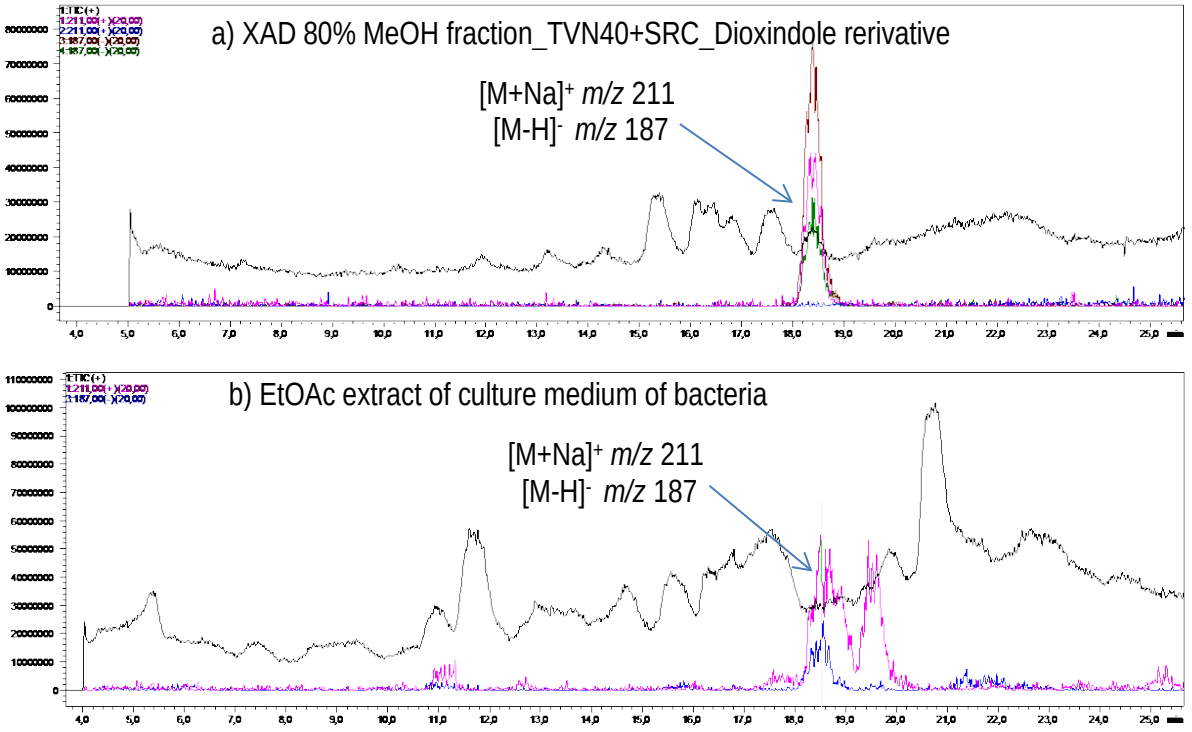


Figure III-58. Mass chromatograms of *m/z* 239 according to $[M+H]^+$ of flourensadiol.

III.6.10.2. *ESI MS analysis for the presence of dioxindole derivative*

Mass chromatograms of the dioxindole derivative were recorded in a scan mode of fraction XAD 80% MeOH of strain TVN40+SRC (see III.6.4.2) are presented below. It is noticed that the presence of *m/z* 211 and 187 values according to $[M+Na]^+$ and $[M-H]^-$ of dioxindole derivative (M = 188) were high in EtOAc extracts of culture medium of bacteria isolated from SRC as well as in TYG medium.



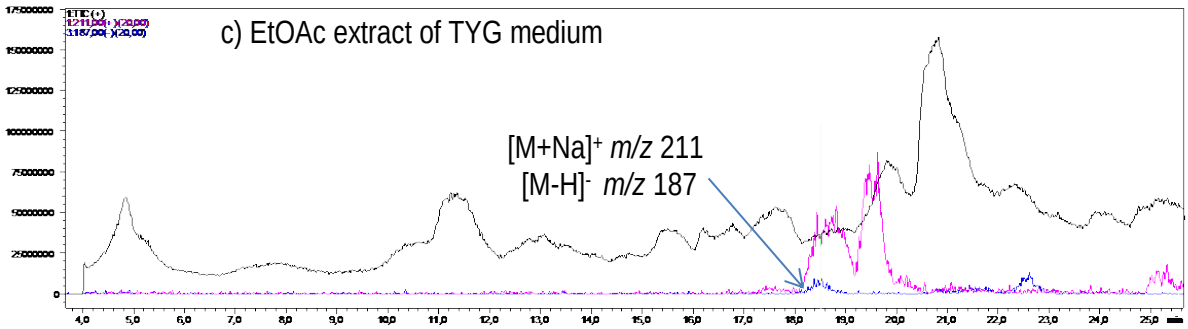


Figure III-59. Mass chromatograms of *m/z* 211 and 187 according to $[M+Na]^+$ and $[M-H]^-$ of dioxindole derivative.

III.6.10.3. *ESI MS analysis for the presence of compound 1, 2 and 5*

Mass chromatograms of compound 1, 2, and 5 recorded in a scan mode of fraction XAD 80% MeOH of strain TVN40+SRC (see III.6.4.2) are presented below. These peaks were not found in all other samples.

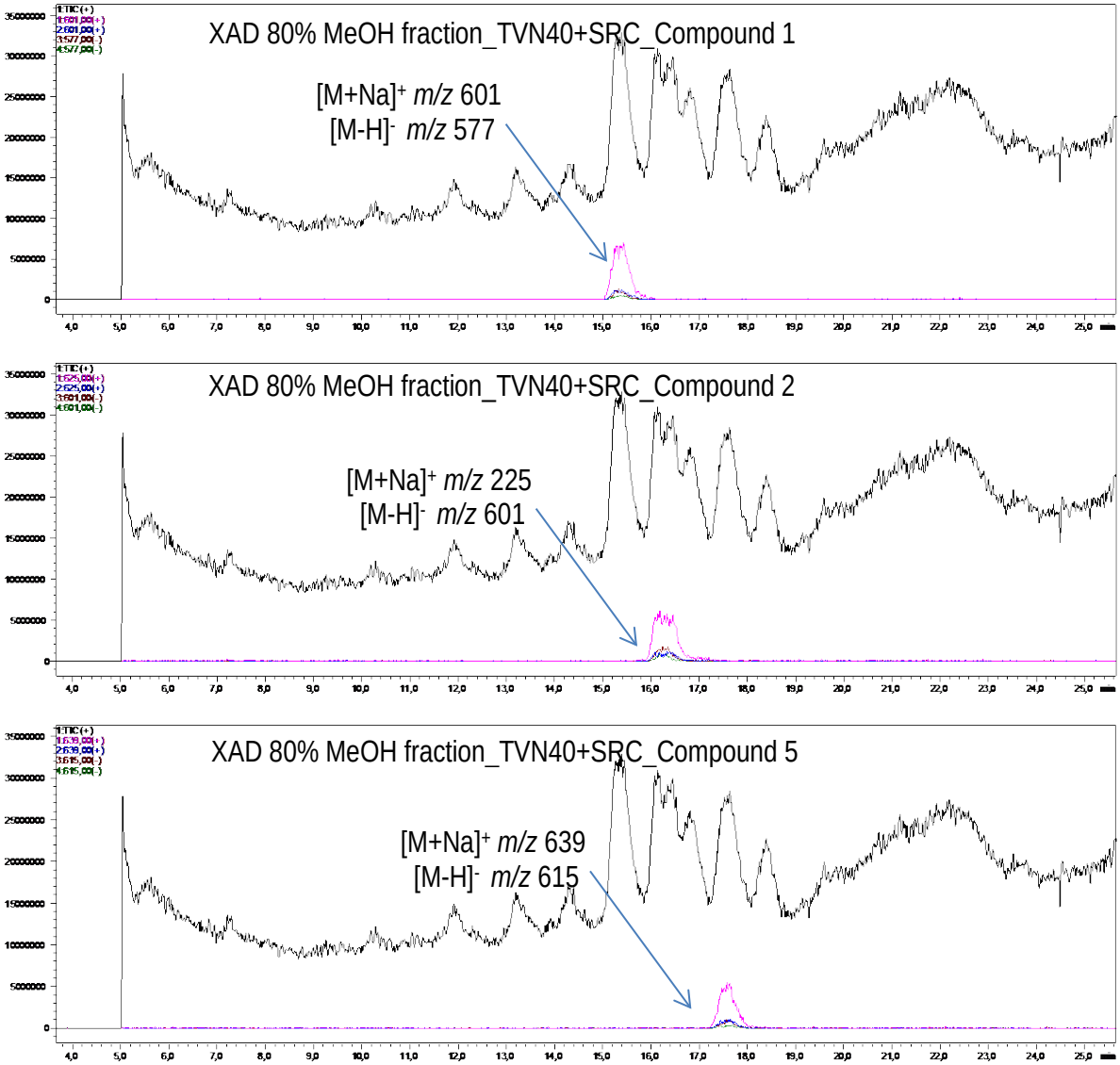


Figure III-60. Mass chromatograms of *m/z* values according to $[M+Na]^+$ and $[M-H]^-$ of compound 1, 2, and 5.

III.7. Axenization of cyanobacteria

III.7.1. Axenization of strain Bio 33, TVN40 and SRC

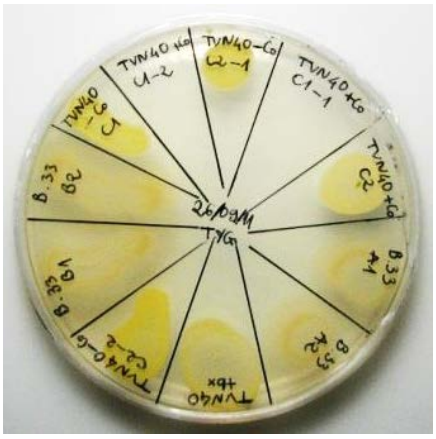


Figure III-61. Testing for the axenic cyanobacteria

Aliquots of 3-week-old isolated cyanobacteria were spread on TYG agar, incubated at 37°C in 48 h. The clear zone indicated the axenic cyanobacterium.

a) b)

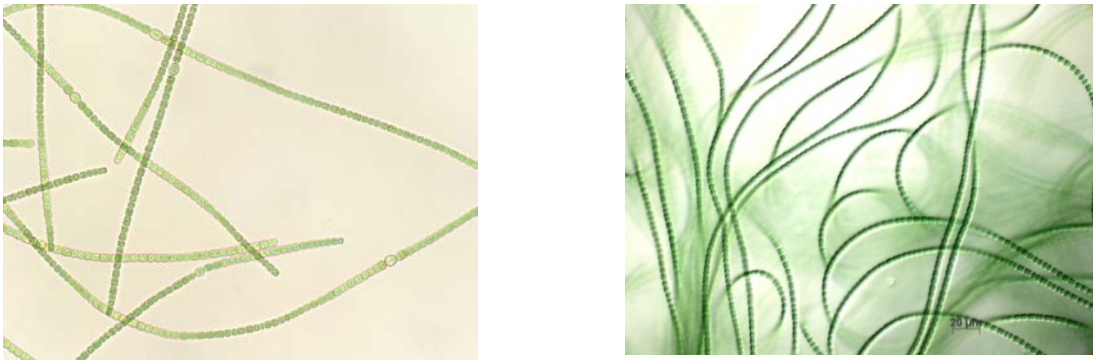
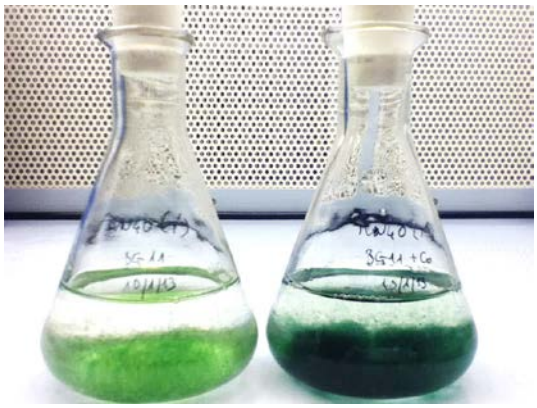


Figure III-62. a) Axenic Bio 33 in BG 11 medium + 0.5% NaCl.
b) Axenic TVN40 in BG 11 medium + Co^{2+}.

The isolated cyanobacterium was axenized with the methods described in II.9. The method using a micropipette to pick up one single filament and wash it several times with distilled medium was successful for strain TVN40 but not for strain Bio 33. However, the method in which the stock culture of Bio 33 was diluted and spread out on the surface of an agar plate was successful for this strain. The SRCs were easily isolated from strain TVN40 using a micropipette but difficult to axenize because of the single form, the small size and the very slow growth rate. The axenic cyanobacteria were checked with TYG plates after 3 weeks of cultivation. As result of axenization, the strains Bio 33 and the filamentous TVN40 are available as axenic cultures. The axenization of SRC for taxonomic identification as well as further isolation the active compounds is still in progress.

III.7.2. Preservation of cyanobacteria in the cultivation

III.7.2.1. *Renewing of isolated cyanobacteria*



a) Strain TVN40+SRC grew in BG 11 (*left*) and BG 11 + Co^{2+} (*right*) media after 4 weeks of cultivation.

b) Non-axenic isolated TVN40 grew in BG 11 (*left*) and BG 11 + Co^{2+} (*right*) media after 4 weeks of cultivation.

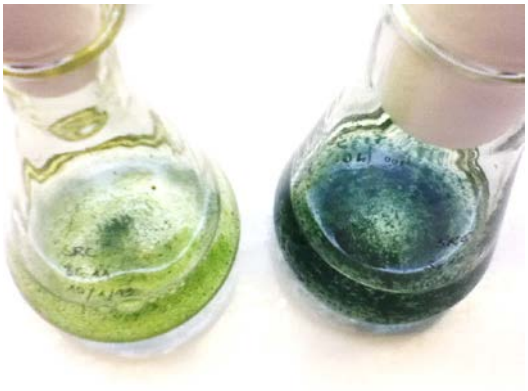


Figure III-63. c) Non-axenic isolated SRC grew in BG 11 (*left*) and BG 11 + Co^{2+} (*right*) media after 4 weeks of cultivation.

In comparison to BG 11 medium, in MBL medium the tested cyanobacteria grew slowly, formed smaller cells and developed clearly yellowish color after 4 weeks, so that MBL medium was not used to preserve the strains. After 5 weeks non-axenic isolates of Bio 33 still grew well in BG 11 + 0.5% NaCl medium. In BG 11 medium without NaCl, the cells were broken so that the culture medium had a bluish

color. Otherwise, Bio 33 filaments grew in BG 11 + NaCl medium with cobalt had a slightly yellowish green color. Therefore, BG 11 + 0.5% NaCl medium was used to renew strain Bio 33 every 4 weeks. 5 mL of old culture medium was transferred into 50 mL of fresh, sterile medium.

Non-axenic mixture of TVN40+SRC as well as non-axenic pure TVN40 and SRC grew well for 5 weeks in BG 11 + Co^{2+} medium and exhibited green color. However, in BG 11 medium without cobalt they developed yellowish green color. Hence, BG 11 medium with cobalt was used to renew these strains after 4 weeks of cultivation (see Figure III-63).

III.7.2.2. *Preservation of axenic cyanobacteria*

Axenic cyanobacteria were placed on the agar surface on 16/09/2011 and were photographed after 2 months (14/11/2011), and 4 months (14/01/2012). Comparison of axenic filaments grew on Bacto agar plates showed that after 4 months axenic Bio 33 (see Figure III-64) still grew well and green in BG 11 plus 0.5% NaCl medium without cobalt. In other media, the filaments were broken and had yellowish color. Therefore, BG 11 medium plus 0.5% NaCl without cobalt was chosen for preservation of axenic Bio 33. The axenic cyanobacterium was able to grow on agar plate containing 25 mL agar for 5 - 6 months.

Although non-axenic isolates of TVN40 grew well in Erlenmeyer flasks containing BG 11 medium with cobalt (see III.7.2.1), the axenic strain died after 2.5 months cultivated on agar plate supplemented with the same medium. Axenic TVN40 did not develop filaments, turned yellow and died after 2.5 months transferred to the agar plate containing BG 11 with Co^{2+} (Figure III-65). Experiments on stock cyanobacteria cultivated in BG 11 medium or BG 11 + Co^{2+} had the same result. It seemed that on agar plates Co^{2+} inhibited the growth of this cyanobacterium. Therefore, agar plates with BG 11 medium without cobalt was used to preserve axenic TVN40.

SRC was preserved in on agar plates with BG 11 medium with cobalt.

14/11/2011 14/01/2012

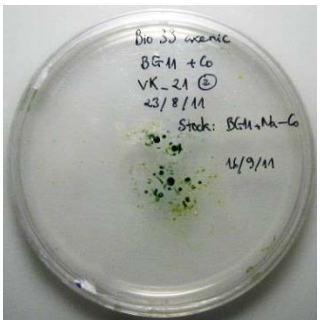
BG 11 + NaCl – Co^{2+}

BG 11 + NaCl – Co^{2+}

BG 11 – NaCl – Co^{2+}

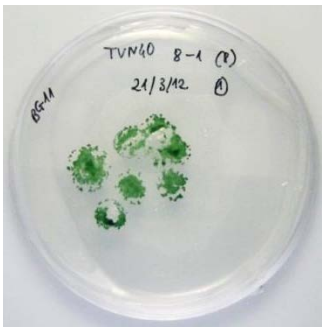
BG 11 – NaCl – Co^{2+}

BG 11 – NaCl + Co^{2+}

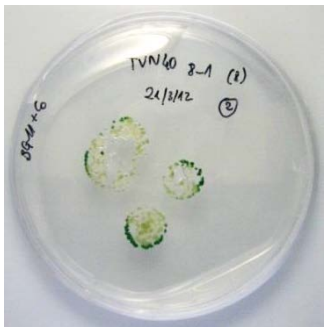


BG 11 – NaCl + Co^{2+}

Figure III-64. Axenic Bio 33 grew on Bacto agar plate supplemented with different media after 4 months of cultivation.



BG 11



BG 11 + Co^{2+}

Figure III-65. Axenic TVN40 grew on Bacto agar plate supplemented with different media after 2.5 months of cultivation.

III.7.3. Antimicrobial activity of the axenic Bio 33

The axenic Bio 33 cultivated in BG 11 plus 0.5% NaCl yielded 0.44 g biomass /L (SD = 0.01, n = 3) medium. In comparison to the non-axenic Bio 33 (0.27 g biomass/L, see III.1.1), the axenic one produced about 2 fold higher biomass. The MeOH/H_2O (1:1) extract of axenic Bio 33 yielded 20.2% of the biomass, lower than that of non-axenic Bio 33 (30.3%, see III.2.1).

All extracts were tested against *Candida maltosa* SBUG 700 (see II.3.3), *Candida albicans* ATCC 90028, *Candida krusei* ATCC 90878, *Aspergillus fumigatus*, *Mucor* sp. (see Appendix I) (2 mg/pd). The results represented in Figure III-66.

Table III-38. Yield of extraction and antifungal activity of axenic Bio 33.

	Mass of extract (%)	Inhibition zone (mm)
n-hexane	2.6	0
MeOH/H_2O	20.2	17.5
H_2O	3.7	0

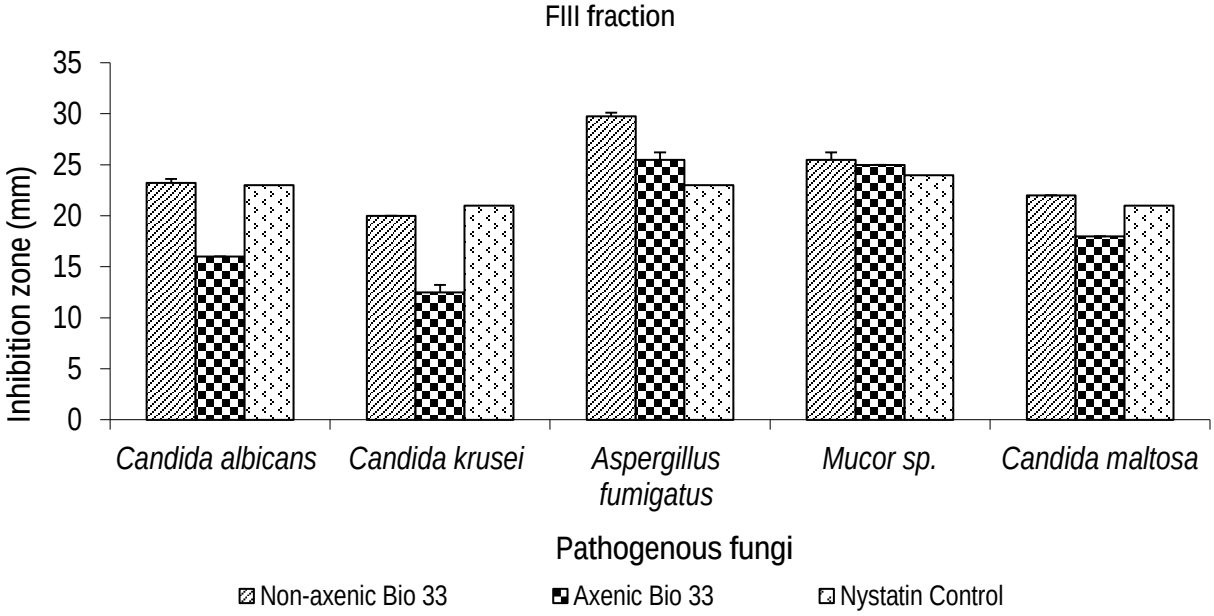


Figure III-66. Antifungal activity of FIII fraction of the axenic and non-axenic Bio 33 against some pathogenous fungi.

Extract concentration 2mg/6mm paper disc, inhibition zone including the diameter of paper disc. Nystatin 10 μg/paper disc. n = 2.

The active compounds from the axenic Bio 33 were analyzed by HPLC according to the method described in II.6.1.7. In HPLC analysis, peak areas of peaks 1, 2 and 3 of non-axenic strain were larger than that of axenic strain (Figure III-67). The same phenomenon was observed in agar diffusion assay when the MeOH/H_2O (1:1) extract of non-axenic Bio 33 displayed larger inhibition zone (22.0 mm) than

the extract of the axenic strain (18 mm) (see III.2.1). In summary, the non-axenic Bio 33 exhibited higher antifungal activity than the axenic one.

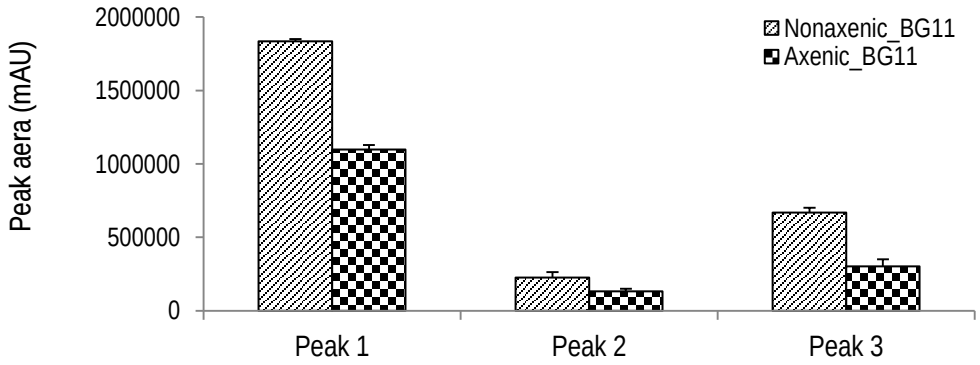


Figure III-67. Peak area of the MeOH/H_2O (7:3) extracts of non-axenic and axenic Bio 33.
Bio 33 was cultivated in BG 11 + 0.5% NaCl. n = 2.

III.8. Taxonomy

III.8.1. 16S rRNA gene sequences of Bio 33, TVN40, SRC and isolated bacteria

Genomes of axenic strains Bio 33 and TVN40 were isolated from cells using ultrasonication and lysozyme. The genes of 16S rRNA were amplified by polymerase chain reaction (PCR) and purified. Cells of SRC and other bacteria were introduced to the PCR without an extra genomic isolation step. After PCR, the purified 16S rDNA samples were estimated with a Nanodrop 1000 as 103.1 and 177.5 ng/μL for strain Bio 33 and TVN40, respectively. These samples were used for sequencing of 16S rRNA gene. The sequences of the partial 16S rRNA genes of investigated strains are presented in Index VIII. These obtained sequences were compared to sequence information available in the National Center for Biotechnology Information database using BLAST analysis (Altschul *et al.*, 1997) (http://www.ncbi.nlm.nih.gov/BLAST).

Table III-39. The partial 16S rRNA gene sequences of strain Bio 33, TVN40, SRC and isolated bacteria from SRC culture.

No.	Strain	Length (nucleotides)
1.	Bio 33	1268
2.	TVN40	1334
3.	SRC	1350
4.	WL1	1353
5.	WS1	1403
6.	Y1	1357
7.	YM1	1377

Four best hits of the blast search of the 16S rDNA sequence of strain Bio 33 showed: *Anabaena cylindrica* PCC 7122 strain CCAP 1403/2A (Indentity 99%)/Accession number HF678516.1; *Anabaena cylindrica* PCC 7122 strain PCC 7122 complete sequence (99%)/NR_102457.1; *Anabaena cylindrica* PCC 7122, complete genome (99%)/CP003659.1; *Anabaena cylindrica* PCC 7122 strain CCAP 1403/2B (99%)/HE975014.1.

Four best hits of the blast search of the 16S rDNA sequence of strain TVN40 showed: *Nostoc* sp. PCC 7107, complete genome (97%)/CP003548.1; *Nostoc* sp. PCC 9426 (98%)/AM711538.1; *Trichormus azollae* Kom BAI/1983 (98%)/AJ630454.1; *Nostoc ellipsosporum* CCAP 1453/2 (97%)/HF678488.1.

Four best hits of the blast search of the 16S rDNA sequence of SRC showed: Chroococcales cyanobacterium LEGE 06123 (99%)/FJ589716.1; *Gloeocapsa* sp. PCC 7428 16S ribosomal RNA, complete sequence (97%)/NR_102460.1; *Gloeocapsa* sp. PCC 7428, complete genome (97%)/CP003646.1; Unicellular thermophilic cyanobacterium tBTRCCn (97%)/DQ471448.1.

Four best hits of the blast search of the 16S rDNA sequence of bacterium 1 (WL1) showed: *Devosia riboflavina* strain HPG62 (99%)/JQ291598.1; Uncultured *Devosia* sp. clone C50 (99%)/JF833669.1; *Devosia riboflavina* strain NBRC 13584 (99%)/AB680451.1; *Devosia riboflavina* strain Foster strain 4R3337 (99%)NR_042171.1.

Four best hits of the blast search of the 16S rDNA sequence of bacterium 2 (WS1) showed: *Hydrogenophaga* sp. TRS-05 (99%)/AB166889.1; *Hydrogenophaga intermedia* strain C1 (99%)/FJ009392.1; *Hydrogenophaga flava* strain: NBRC (98%)/AB681848.1; *Hydrogenophaga intermedia* strain: NBRC (98%)/AB681844.1.

Four best hits of the blast search of the 16S rDNA sequence of bacterium 3 (Y1) showed: Uncultured *Sphingomonas* sp. clone W4S45 (99%)/GU560166.1; *Sphingopyxis* sp. M2R2 (99%)/EU188914.1; *Sphingomonas* sp. NBRC 15915 (99%)/AB680998.1; *Sphingopyxis alaskensis* strain RB2256 (99%)/NR_074280.1.

Four best hits of the blast search of the 16S rDNA sequence of bacterium 4 (YM1) showed: *Rhodococcus* sp. MDT1-11 (99%)/JX949628.1; *Rhodococcus* sp. P11-B-8 (99%)/EU016150.1; *Rhodococcus* sp. SGB1168-118 (99%)/AB010908.1; *Rhodococcus* sp. SGB1168-116 (99%)/AB010907.1.

According to the blast search, Bio 33 was identified as *Anabaena cylindrica* belonging to order Nostocales; TVN40 was identified as *Nostoc* sp. belonging to order Nostocales; SRC was identified

as cyanobacterium *Gloeocapsa* sp. belonging to order Chroococcales. Four bacteria WL1, WS1, Y1 and YM1 were identified as: *Devosia riboflavina, Hydrogenophaga* sp, *Sphingomonas* sp., and *Rhodococcus* sp.

III.8.2. Phylogenetic relationship of strain Bio 33

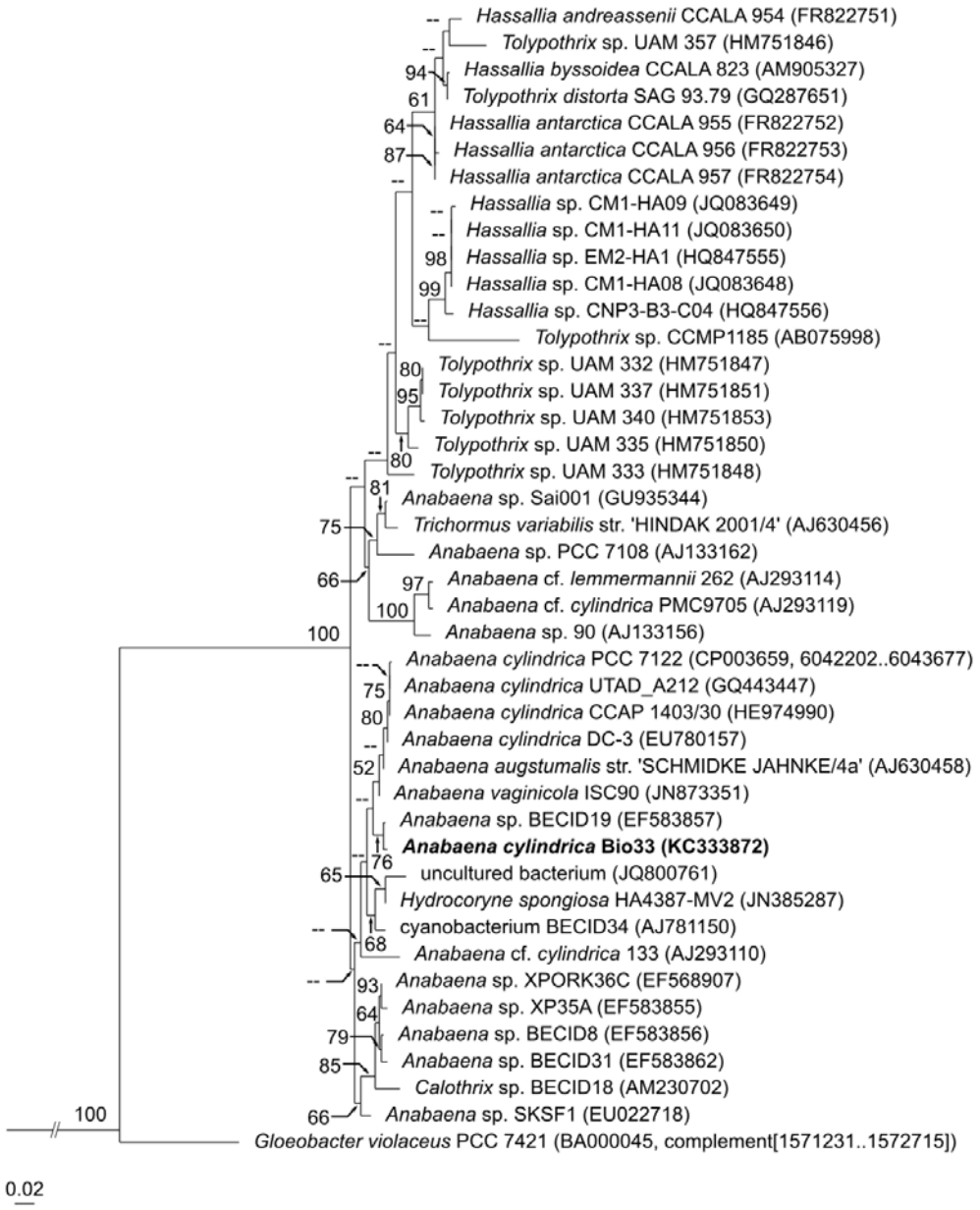


Figure III-68. Phylogenetic relationship of axenic filamentous *Anabaena cylindrica* Bio 33 based on 16S rRNA sequence analysis.

Phylogenetic tree based on a secondary structure alignment containing 46 partial 16S rRNA gene sequences. The tree was inferred using a maximum likelihood method. Numbers given on the branches display bootstrap proportions as percentage of 1000 replicates for values greater than or equal to 50%. The accession numbers of sequences used for reconstruction are given in parentheses. For better clarity, sequences used as outgroup (*B. subtilis* (AJ276351), *E. coli* (DQ360844)) are not shown.

In order to infer the phylogenetic position of strain Bio 33 the nearly complete 16S rRNA gene of Bio33 was used for construction of a phylogentic tree using a maximum likelihood method. The final tree was the best tree out of 1000 independent inferences (Figure III-68). The sequence of strain Bio33 was included into the INSDC databases, i.e. DDBJ, EMBL, Genbank, under accession number KC333872.

IV. Discussion

IV.1. Screening

With regard to increasing invasive fungal diseases and rising antifungal resistance, the current antifungal drug development is not adequate to combat fungal diseases. Thus, the rising need for antifungal agents, particularly those for the treatment of systemic fungal infections, always promote the pharmaceutical research, especially the search for more potent antifungal substances.

The common method used for antimicrobial screening of intra- and extracellular extracts from cyanobacteria is based on the agar diffusion assay with selected microorganisms as test organisms (Cannell *et al.*, 1988b; Pawar *et al.*, 2008; Medina-Jaritz *et al.*, 2011). For evaluation of the antifungal acitivity of extracts, it is advantageous to classify the activity according to the inhibition zones into four levels: low activity (IZ = 7-10 mm), moderate activity (IZ = 11-15 mm), strong activity (IZ = 16-20 mm) and very strong activity (IZ > 20 mm).

In our screening, 133 intra- and extracellular extracts (including 36 *n*-hexane extracts, 43 MeOH extracts, 35 H_2O extracts and 19 EtOAc extracts) from 28 cyanobacterial strains were tested against *C. maltosa*, *P. aeruginosa*, *E. coli*, *B. subtilis* and *S. aureus* using agar diffusion assay. As a result, 30 extracts of 133 tested extracts (22.6%) from 13 cyanobacterial strains (46.4%) exhibited antifungal activity against *C. maltosa*, mostly from low to moderate activity. Strong activity was observed only with two MeOH extracts of *Anabaena* sp. strain Bio 33 and *Nostoc* sp. strain 10. Meanwhile, there were 12 extracts (9.0%) from 10 strains (35.7%) showed positive activity against *B. subtilis* and 13 extracts (9.8%) from 11 strains (39.3%) showed positive activity against *S. aureus*. Most of them showed low to moderate activity. Strong activity was observed from 5 extracts (3.8%). Only the extract of the cultivation medium (0.75%) from *Anabaena* sp. strain TVN40 (3.6%) was active against the Gram negative bacteria *P. aeruginosa* and *E. coli*. Almost all of these positive results were found in *n*-hexane and methanol extracts although the intensity of the inhibitory effects was variable. Only three aqueous extracts of strains Bio 33, CAVN 11 and CAVN 20 exhibited low to moderate antimicrobial activity.

As far as known, cyanobacterial extracts in published screenings showed antifungal activity with a proportion between 12-50% (Klochenko *et al.*, 2001; Ördög *et al.*, 2004; Soltani *et al.*, 2005; Kim, 2006; Pawar *et al.*, 2008). Moreover, different screening methods can lead to dissimilar results due to different sensitivities of the methods and permeabilities of bioactive substances into the test organisms (Pawar *et al.*, 2008). Up to date, extracts of cyanobacteria grown in the laboratory were

commonly found to exhibit antibacterial activity rather than antifungal activity. Furthermore, activity against Gram-positive bacteria has been frequently observed but activity against Gram-negative bacteria is rare (Pedersén *et al.*, 1973; Cannell *et al.*, 1988b; Moore, 1996; Kreitlow *et al.*, 1999; Mundt *et al.*, 2001; Svircev *et al.*, 2008; Medina-Jaritz *et al.*, 2011). Besides, cyanobacterial intra- and extracellular extracts (Bui, 2006; Le, 2010) as well as secondary metabolites such as comnostins (Jaki *et al.*, 2000) and noscomin (Jaki *et al.*, 1999) which are active against Gram negative bacteria were already reported. Moreover, antibacterial activity was especially found in the more unpolar and middle polar extracts while the aqueous extracts were ineffective normally (Mundt *et al.*, 2001). In comparison with the literatures, the same phenomenon was observed in our screening when there are more extracts exhibiting activity against Gram-positive bacteria than Gram-negative bacteria. However, more strains in our screening were found to possess antifungal activity than antibacterial activity. As mentioned in many publications, biological activity of cyanobacteria varied between tested species and between strains of the same species. Normally, the cyanobacteria were harvested in their early stationary phase of growth to minimize some of these variations (Ördög *et al.*, 2004). It is needed for further investigation to be sure about the origin of the activity: inherent strain differences, different stages of growth of the cyanobacteria when harvested, or culture conditions.

The ecological role of bioactive molecules in the cyanobacterial producer itself is still poorly understood (Martins *et al.*, 2008). Nevertheless, cyanobacteria belong to the Gram-negative bacteria, therefore their metabolites have stronger activity against gram positive bacteria and yeasts (Mundt *et al.*, 2001). This fact supports the assumption that these products can be produced to protect cyanobacteria themselves from competing organisms (Piccardi *et al.*, 2000; Mundt *et al.*, 2001; Bhadury *et al.*, 2004; Martins *et al.*, 2008).

Among the crude extracts which were positive against *C. maltosa*, those of strain Bio 33 exhibited strong and stable activity with inhibition zone of 20 mm (2 mg/paper disc). To clarify the specificity of this effect, the extracts were further tested against other bacteria. It was very impressed that the crude extracts of strain Bio 33 had no antibacterial effect against two Gram-positive bacteria (*B. subtilis* and *S. aureus*) and two Gram-negative bacteria (*E. coli* and *P. aeruginosa*). There were many available antifungal compounds whose activity is a consequence of their unspecific cytotoxicity such as the scytophycins (Jung *et al.*, 1991) and laxaphycins (Gbankoto *et al.*, 2005). Therefore, the selective activity of the crude extract from strain Bio 33 was highly appreciated.

As a result of our screening, strains Bio 33 and TVN40 with interesting antifungal activity were chosen for isolation of bioactive compounds.

IV.2. Cyanobacterial research in the Baltic Sea

Since 1960s, when water blooms were in clear evidence in the Baltic Sea (Finni *et al.*, 2001), the plankton research within this area has been intensively expanded. Many researches indicated that three filamentous nitrogen-fixing cyanobacteria *Nodularia*, *Aphanizomenon*, and *Anabaena* are dominant genera which cause water blooms in the Baltic Sea during the warmest summer months (Sivonen *et al.*, 1989b; Barker *et al.*, 1999; Laamanen *et al.*, 2001; Laamanen *et al.*, 2002). However, the Baltic Sea cyanobacterial reports have mostly focused on the toxins produced by *Nodularia* and *Aphanizomenon* (Sivonen *et al.*, 1989a; Congestri *et al.*, 2000; Lehtimaeki *et al.*, 2000; Finni *et al.*, 2001; Laamanen *et al.*, 2001; Janson *et al.*, 2002; Stal *et al.*, 2003; Mohlin, 2010). It was mentioned by Sivonen that the nodularin concentration in the Baltic Sea was 18 mg g^{-1} of biomass dry weight – the highest concentration measured in nature (Jones *et al.*, 1999; Sivonen *et al.*, 2007). In contrast, even though the freshwater *Anabaena* strains in Canada, Denmark, Egypt, Finland, France, and Norway have been identified as Microcystin-producer (Jones *et al.*, 1999; Halinen *et al.*, 2007), the *Anabaena* spp. from the Baltic Sea has been overlooked in earlier cyanobacterial studies due to presumptions about their nontoxicity (Sivonen *et al.*, 1989a; Sivonen *et al.*, 2007). Nevertheless, recent researches identified *Anabaena* in the Baltic Sea as a hepatotoxins (i.e. microcystins) (Karlsson *et al.*, 2005; Halinen *et al.*, 2007) or cytotoxins producer (Surakka *et al.*, 2005).

In the screening program for antibiotic activity of cyanobacteria, the group of S. Mundt (Kreitlow *et al.*, 1999) found that the water extract of water bloom samples collected from different regions of the Baltic Sea during the summer of 1997 showed activity against *Staphylococcus aureus*. The water bloom sample consisted mainly of *Microcystis ichthyoblabe*, *Anabaena lemmermannii* and *Nodularia* sp.. In addition, there were no inhibitory effects against the yeast *Candida maltosa* (Kreitlow *et al.*, 1999). Another report of this group in 2001 also indicated that the aqueous extract of the water bloom sample SPH 01 collected from the Baltic Sea, which contained *Microcystis aeruginosa*, showed antiviral activity against influenza A virus without any cytotoxic effects against the host cells (Mundt *et al.*, 2001). The cyclic and linear peptides, which are protease inhibitors, produced by *Microcystis aeruginosa* strains (Namikoshi *et al.*, 1996) were assumed for the antiviral effects (Mundt *et al.*, 2001). Up to now, there is no available report about antifungal compounds produced by cyanobacteria from the Baltic Sea.

The genus *Anabaena* is one of the most dominant components of planktons in freshwater lakes, ponds, reservoirs, and in brackish water bodies worldwide. *Anabaena*, together with *Oscillatoria*, *Microcystis*, *Nodularia*, *Cylindrospermopsis* and *Lyngbya* are the cyanobacterial genera which have toxicological or pharmacological significance (Liu *et al.*, 2010). The biological activities published so far for *Anabaena* genus belong mostly to two major groups: cytotoxicity or enzyme inhibition (Table

IV-2). Hepatotoxins such as microcystins, and neurotoxins such as anatoxin-a, anatoxin-a(S), and saxitoxins are the common toxins found in freshwater *Anabaena* spp. (Jones *et al.*, 1999; Beltran *et al.*, 2000).

Table IV-1. *Anabaena* spp. occur in the Baltic Sea at German subarea

		Synonym	KB	AB
Anabaena Bory *ex* Bornet & Flahault 1886	*affinis* Lemmermann 1897	[*Anabaena catenula* v. *affinis* (Lemmermann) Geitler 1932]	-	f
	baltica J. Schmidt 1899	[*Anabaena spiroides* f. *baltica* (J. Schmidt) Pankow 1965]	w	w
	cylindrica Lemmermann 1896	[*Anabaena subcylindrica* Borge 1921]	+	+
	flos-aquae (Lyngbye) Brébisson *in* Brébisson & Godey *ex* Bornet & Flahault 1886	[*Anabaena contorta* Bachmann 1921]	f	f
	inaequalis Kützing *ex* Bornet & Flahault 1886		+	+
	lemmermannii P. Richter *in* Lemmermann 1903	[*Anabaena flos-aquae* f. *lemmermannii* (P. Richter) Canabaeus 1929] [non *Anabaena flos-aquae* auct. plur.]	+	w
	spiroides Klebahn 1895		wef	wf

Data adopted from Hällfors (2004).

KB Kattegat and the Belt Sea area

AB Arkona Basin

+ occurrence without ecological characterization

w warm water species

f freshwater species which does not tolerate the full salinity of the area

s species belonging to waters of higher salinity than that of the area

e main occurrence in eutrophied waters

− no record

Additionally, the genus *Anabaena* was also found to exhibit fungicidal activity. In a screening program of Prasanna *et al.*, 28 *Anabaena* strains isolated from diverse agro-ecologies of India (Nayak *et al.*, 2007) showed allelopathic and fungicidal activities (Prasanna *et al.*, 2006; Prasanna *et al.*, 2008). A later study of Gupta *et al*. on these 28 *Anabaena* strains using molecular approaches found six strains producing microcystin toxins (Gupta *et al.*, 2012).

Table IV-2. Some natural products isolated from cyanobacteria *Anabaena* spp.

Source	Name	Activity	Class of compound	Reference
Anabaena laxa	Laxaphycins A and B	synergistically antifungal	Cyclic peptide	(Frankmölle *et al.*, 1992a; Frankmölle *et al.*, 1992b)
Anabaena cylindrica	Dehydroradiosumin	trypsin inhibitor	Peptide	(Kodani *et al.*, 1998)
	Circinamide	neurotoxin, papain inhibitor	Alkaloid	(Shin *et al.*, 1997b)
	Anabaenopeptins	inhibit protein phosphatase	Peptide	(Harada *et al.*, 1995)
Anabaena sp.	Microcystins, puwainaphycin	cardioactive, hepatotoxin	Lipopeptide	(Carmichael *et al.*, 1990; Nishiwaki-Matsushima *et al.*, 1992)
Anabaena circinalis	Circinamide	neurotoxin, papain inhibitor	Alkaloid	(Negri *et al.*, 1995; Shin *et al.*, 1997b; Beltran *et al.*, 2000)
	Anatoxin-a	neurotoxin	Alkaloid	(Devlin *et al.*, 1977)
	Saxitoxins	neurotoxin	Alkaloid	(Wiese *et al.*, 2010)
	Microcystin	enzyme inhibitor	Lipopeptide	(Sim *et al.*, 1993)
	Anabaenopeptins	inhibit protein phosphatase	Cyclicpeptide	(Harada *et al.*, 1995)
	Microcystins A, B, and C	hepatotoxin	Lipopeptide	(Sim *et al.*, 1993; Namikoshi *et al.*, 1996)
Anabaena flos-aquae	2;9-Diacetyl-9-azabicyclo(4;21)non-2;3-ene, siatoxin	toxic, antibiotic, anticancer	Lipopeptide, alkaloid	(Burja *et al.*, 2001)
	Saxitoxin (aphantoxins)	neurotoxin	Alkaloid	(Carmichael *et al.*, 1990)
	Microcystins A, B, and D	hepatotoxin	Lipopeptide	(Harada *et al.*, 1991; Namikoshi *et al.*, 1996)
	Anatoxin-a(s)	neurotoxin	Alkaloid	(Mahmood *et al.*, 1988; Matsunaga *et al.*, 1989; Carmichael *et al.*, 1990; Harada *et al.*, 1995)
	Anabaenopeptins A and B	caused relaxations in rat aortic preparations,	Cyclic peptide	(Harada *et al.*, 1995)

		inhibit protein phosphatase		
Anabaena variabilis	Bis(χ-butyrolactones)	antibiotic	Lactone	(Carmichael *et al.*, 1975; Burja *et al.*, 2001)
	Plastocyanin	physiological redox reagent	Copper protein	(Lightbody *et al.*, 1967)
Anabaena sp.	Diarrhetic toxin	cytotoxic	Lipopeptide	(Burja *et al.*, 2001)
Anabaena lemmermannii	Anabaenopeptins B and D	no animal toxicity or inhibition of protein phosphatases.	Cyclic peptide	(Harada *et al.*, 1995)
	Microcystins B, C, and D	hepatotoxin	Cyclic peptide	(Namikoshi *et al.*, 1996)
	Anatoxin-a(s)	neurotoxin	Alkaloid	(Onodera *et al.*, 1997)
Anabaena strain 90	Anabaenopeptilide 90A and 90B	no activity	Cyclic depsipeptide	(Rouhiainen *et al.*, 2000)

Adapted from Namikoshi *et al.* (1996).

IV.3. Isolation and characterization active compounds from Bio 33

The dried biomass of cyanobacteria was separated based on a previously reported procedure (Kreitlow *et al.*, 1999) into un-polar (*n*-hexane extract), middle polar (methanol extract) and polar (aqueous extract) crude extracts. Normally, the active compounds (i.e. polyphenolic compounds, terpenoids, alkaloids, peptides) are concentrated in the methanol crude extract. However, the methanol extract and the aqueous extract of strain Bio 33 both exhibited inhibitory effect against *C. maltosa*. This observation suggested that the active compound(s) of this strain should be rather polar. Thus, the combination of methanol/water (1:1) was used after *n*-hexane to extract all of the active compounds out of the biomass. The polarity of the active compounds was later proved by silica gel thin layer chromatography, where the active band moved only by adding water to the solvent system. Moreover, when comparing the inhibition zone on the TLC plate found in the autobiographic assay with the bands on the control TLC (sprayed with ninhydrin, anisaldehyde and 20% H_2SO_4 in water reagents), we suggested that there were at least four compounds containing peptides in their structures.

For fractionation of the crude extract, a silica gel normal phase chromatography column with a mobile phase containing a solvent system of ethyl acetate, methanol, and water in different ratio was used. Although the substances such as peptides and compounds with polar functional groups would be retained by silica gel and eluted later, chlorophyll and most of other pigments as well as undesired

compounds (about 70%, see III.2.5) were separated from the main active fraction. After being separated into 4 fractions by the open column, the main fraction FIII of the methanol/water (50:50) crude extract exhibited a remarkable antifungal activity. Further separation of the main fraction FIII was carried out with SPE C18 cartridges. The majority of impurities such as inorganic materials and polar contaminants have been easily removed by washing the cartridge with 20% aqueous methanol.

Because the autobiography resulted in closely active bands of the main fraction FIII as well as the SPE fractions, HPLC was applied to separate the pure active compounds. C18-silica phase are generally preferred for hydrophilic peptides and small proteins less than about 5000 daltons due to its efficiency at adsorbing peptides (Gupta *et al.*, 2011). Most RP-HPLC phases interact with analytes via weak van der Waals or dispersive interactions, which are due to an overlap of the outer electron clouds between the analyte and bonded phase. RP-HPLC mobile phases for peptide separation are usually gradients of an organic modifier (ACN or MeOH) in water, with an ionic modifier that controls the pH and ionization state or acts as an ion pair agent. Thus, different C18 RP-HPLC columns with MeOH/H_2O or ACN/H_2O as mobile phase were used to separate the mixture but a successful separation was only reached when TFA was added to the H_2O mobile phase. This fact once again suggested that the active compounds might be peptides.

At low concentration, TFA can change the selectivity by improving the interaction between peptides and the stationary phase in liquid chromatography of organic compounds, particularly peptides and small proteins, due to its ion pairing capacity (binding by ionic interaction to the solute molecules to increase the hydrophobicity of the solute molecule). The functions of TFA can be: pH control, ion pairing (complexation with oppositely charged ionic groups) to enhance reversed phase retention, or suppression of silanol activity (adverse ionic interactions between peptides and silanol groups on the silica). The latter function is necessary when using RP-HPLC phases with high silanol activity (Rogers *et al.*, 2000). Hence, TFA has been long used as an additive in reversed-phase HPLC for separation of proteins and peptides (McCalley, 2005). A concentration range of 0.05 – 0.1% or 50 - 100 mM is commonly used (pH 2 – 3). Nevertheless, varying the concentration of TFA may affect selectivity or resolution of specific peptide pairs. Thus, for good reproducibility in peptide separation methods, the TFA concentration must be controlled carefully. After the column and gradient conditions were selected, the TFA concentration was varied to further optimize resolution between peaks and a TFA concentration of 0.05% in water was chosen for separation the antifungal compounds of strain Bio 33.

As a result of the HPLC purification, four pure compounds named balticidins A-D have been isolated as white solids. The structure elucidation of these compounds led to lipopeptides represent a structural type with co-occurrence of a glycosylated (mannose is attached) aliphatic (A and C) or

cyclic peptide core (B and D), linked with a C16 fatty acid to which a disaccharide moiety, composed of galacturonic acid and arabinose, is bound. Balticidins A and B contain chlorine in the aliphatic fatty acid residue. The chlorine is absent in C and D.

A literature study revealed two closely related cyclic peptides, hassallidin A and B (see page 19) (Neuhof *et al.*, 2005; Neuhof *et al.*, 2006), isolated from an epilithic cyanobacterium collected in Bellano, Italy, identified as *Tolypothrix* (basionym *Hassallia*) species. This species belongs also to the order Nostocales but to the family Microchaetaceae. The already known hassallidins are esterified eight-residue cyclic peptides linked with a fatty acid residue – dihydroxytetradecanoic acid and possess antifungal activity. Hassallidin A contains the sugar mannose, hassallidin B differs from A by an additional carbohydrate unit - rhamnose, attached to the anomeric carbon position 3 of the fatty acid (Neuhof *et al.*, 2005; Neuhof *et al.*, 2006). Our data are compatible with those observed for hassallidin A, in which chiral GC-MS analysis revealed the occurrence of the nonproteinogenic amino acids D-*allo*-Thr, D-Thr, D-Tyr, D-Gln, and dehydroaminobutyric acid (Dhb) within the peptide moiety. The absence of reference material prevented the analysis of the other amino acid residues present but we have now elucidated the position of the D and L-forms of Gln in the peptides. In comparison to the structure of hassallidin A and B, our four structures have the same amino acid sequence as them with a mannose attached to ThrNMe-8, except for OHTyr-4 taking the place of Tyr-4. The IR spectrum of balticidin B showed absorption at 1737 cm^{-1} close to the absorption given for hassalidin A (1740 cm^{-1}) and typical for the presence of an ester/lactone structure which was absent in the linear peptides balticidin A and C. Besides, balticidins contain a dihydroxyhexadecanoid acid side chain and a disaccharide composed of D(-)-arabinose and D(+)-galacturonic acid linked to the 3-OH of the fatty acid residue. In addition, the occurrence of chlorine in the fatty acid unit of balticidin A (1) and B (2) is a unique difference between these compounds and the hassallidins. On the other hand, structural analysis revealed 1 and 3 as linear lipopeptide analogs of 2 and 4. Till now, the balticidins are the first antifungal cyanobacterial lipopeptides containing an oligosaccharide moiety attached to a halogenated fatty acid.

According to a review of Wright *et al.* (Ghazala *et al.*, 2005) which represented major types of cyanopeptide metabolites, glycosylated cyclic lipopeptides such as hassallidins belonged to the cyclic hybrid depsipeptides group. In 2006, based on the common molecular structures of around 600 published cyanobacterial peptides, Welker and von Döhren classified cyanopeptides into 7 groups: aeruginosins, microginins, anabaenopeptins, cyanopeptolins, microcystins, microviridins, and cyclamides (Welker *et al.*, 2006). Nevertheless, together with hassallidins, balticidins B and D represent a new antifungal group of cyanobacterial peptides, which possesses an esterified eight-

residue cyclic peptide linked with carbohydrate and fatty acid residues. In addition, aliphatic chlorination has been reported for microginins (Ishida *et al.*, 1998) but aliphatic chlorinated cyanobacterial peptides linked with oligosaccharide residues like balticidins A and C have not been isolated from cyanobacteria before.

These four antifungal lipopeptides were isolated from the Baltic Sea cyanobacterium *Anabaena* strain Bio 33 belonging to order Nostocales, family Nostocaceae. Possibly, the linear balticidin A (1) and C (3) could be dehydration products of the balticidin B (2) and D (4). Their biosynthesis pathway has not been clarified so far but it is implausible, that the aliphatic compounds are artefacts produced during isolation process for example by adding TFA to the mobile phase – used for separation of the substances. The TFA concentration was only 0.05%, a concentration normally used for separating different cyclic peptides (Lawton *et al.*, 1994; Chuang *et al.*, 2008; Matthew *et al.*, 2009; Bucar *et al.*, 2013). Furthermore, HPLC analysis of the pure compounds also using TFA in the mobile phase revealed only the peak of the injected compound and no decompensation product.

The fact that the same or very similar compounds are produced by two different cyanobacteria belonging to two different families or two different orders is often observed in cyanotoxins. For instance, the hepatotoxin Microcystin was produced by *Oscillatoria* sp. (order Nostocales, family Oscillatoriaceae), *Anabaena* sp. (order Nostocales, family Nostocaceae), *Nostoc* sp. (order Nostocales, family Nostocaceae), *Microcystis* sp. (order Chroococcales), or *Hapalosiphon* sp. (order Stigonematales) (classification according to Rippka *et al.* 1979).

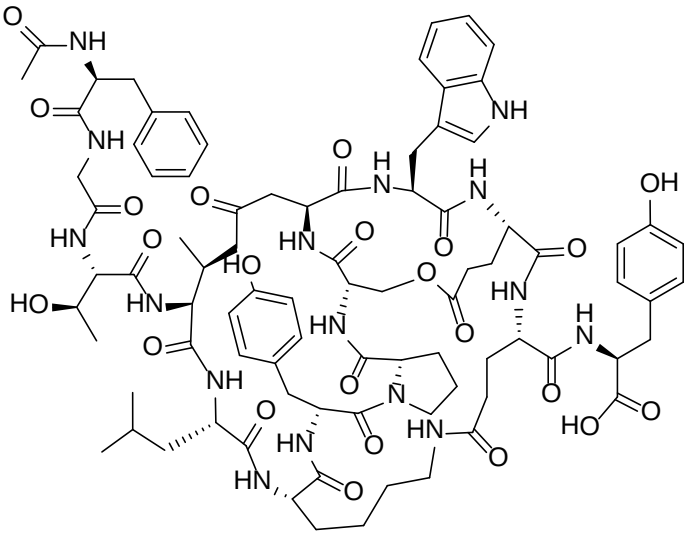
Antimicrobial activity of cyanobacteria refers mostly to filamentous strains belonging to a wide range of genera in orders Nostocales, Oscillatoriales, Stigonematales, but also in Pleurocapsales (Burja *et al.*, 2001). However, orders Oscillatoriales and Nostocales, together with Chroococcales are three orders that produced the vast majority of toxic compounds (Burja *et al.*, 2001). Besides, most toxins observed in the order Nostocales were produced by *Anabaena* and *Nostoc* sp. (Burja *et al.*, 2001; Laurienzo, 2010). In addition, the cytotoxic activity of these biotoxins are responsible for the associated activities, such as anticancer, antifungal, antimalarial, anti-HIV and antimicrobial (Burja *et al.*, 2001). Some examples mentioned in a study of Patterson *et al.* (1991) included acutiphycins, indolcarbazoles, mirabilene isonitriles, paracyclophanes, scytophycins, tantazoles, tolytoxin, toyocamycin and tubercidin (Moore *et al.*, 1990; Carmichael, 1992).

Certain species of the genus *Anabaena* living in both fresh and saltwater is probably known as a neurotoxins producer (see Introduction). At present, neurotoxins anatoxin-a (Harada *et al.*, 1989), homoanatoxin-a (Furey *et al.*, 2003), anatoxin-a(s) (Matsunaga *et al.*, 1989), saxitoxins (Wiese *et al.*, 2010) and neosaxitoxin (Rodriguez-Navarro *et al.*, 2007; Wiese *et al.*, 2010) have been well

described. Previously, planktic *Anabaena* strains isolated from the Baltic Sea were presumed nontoxic, whereas benthic *Anabaena* strains were reported to be hepatotoxic or cytotoxic but not microcystin producing (Herfindal *et al.*, 2005; Surakka *et al.*, 2005; Sivonen *et al.*, 2007). Since 2005, *Anabaena* sp. from the open sea area of the Baltic Sea was reported to produce the hepatotoxin microcystin-LR (Karlsson *et al.*, 2005; Halinen *et al.*, 2007; Halinen *et al.*, 2008).

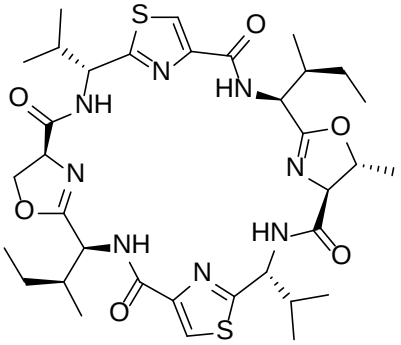
Amongst different forms of cyanobacterial secondary metabolites, peptides are the major part. So far, over 600 variants of cyanobacterial peptides have been described, but the real peptide diversity in cyanobacteria is still beyond the estimation (Welker *et al.*, 2006). About 20 – 75% of the blooms were found to be toxic and the two most commonly isolated groups of cyanotoxins are the alkaloid neurotoxins and cyclic peptide hepatotoxins (Burja *et al.*, 2001). As in the previous review, the majority of 300 discovered marine cyanobacterial nitrogen-containing secondary metabolites covering literature from January 2001 to December 2006 were lipopeptides (Tan, 2007). Some of them are neurotoxic such as antillatoxin A and B (Orjala *et al.*, 1995; Nogle *et al.*, 2001), kalkitoxin (Wu *et al.*, 2000), jamaicamide (Ramaswamy *et al.*, 2001; Edwards *et al.*, 2004; Aráoz *et al.*, 2010), which were isolated from marine cyanobacterium *Lyngbya majuscula*. However, lipopeptides, represented by two structural classes the echinocandins (see I.1) and aureobasidins (Matsunaga *et al.*, 2005) have become a novel class of antifungal agents. The echinocandins are already in clinical use while their semisynthetic derivatives and aureobasidins (Prasanna *et al.*, 2010), are currently under critical investigation. When cyclic peptides isolated from cyanobacteria are commonly cytotoxic and the need for safe and effective antifungal drugs is still rising, cyanobacterial lipopeptides having only marginal cytotoxicity like balticidins may have potency to become usable antifungal agents in therapy.

Peptides produced by cyanobacteria consist of standard as well as modified D- and L-amino acid forms (Welker *et al.*, 2006; El Omari, 2011). Cyanobacterial cyclic and aliphatic peptides which are halogenated at aromatic amino acids were already described (Welker *et al.*, 2006; Rounge *et al.*, 2007) and chlorine was found in 22% of cyanobacterial metabolites (Guyot *et al.*, 2004). Some of them are anabaenopeptilide 90B from *Anabaena* strain 90 (Rouhiainen *et al.*, 2000), cyanopeptolin 954 produced from *Microcystis* NIVA-CYA172/5 (Tooming-Klunderud *et al.*, 2007), cryptophycin 1 from *Nostoc* sp. ATCC 53789 (Chaganty *et al.*, 2004), aeruginosin 98-A, -B, -C from *Microcystis aeruginosa* NIES-98 (Ersmark *et al.*, 2008). Cyanobacterial peptides containing common sugars have rarely been reported, such as hassallidin A (contains mannose, (Neuhof *et al.*, 2005)), hassallidin B (contains mannose and rhamnose, (Neuhof *et al.*, 2006)), aeruginosins 205A and –B (contain xylose, (Shin *et al.*, 1997a)), and suomilide (contains glucose, (Fujii *et al.*, 1997)).



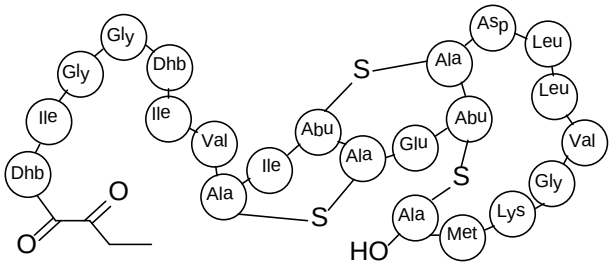
Microviridin B

44

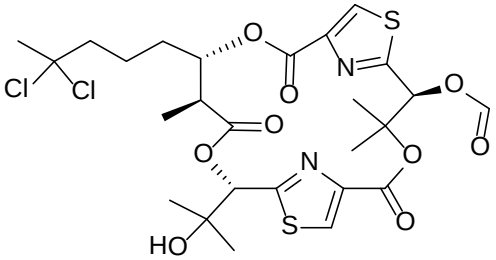


Patellamide A

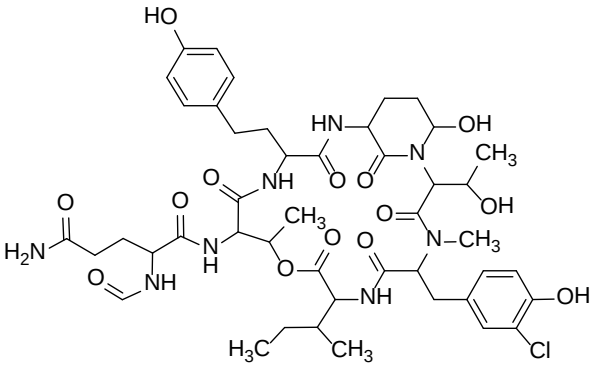
45



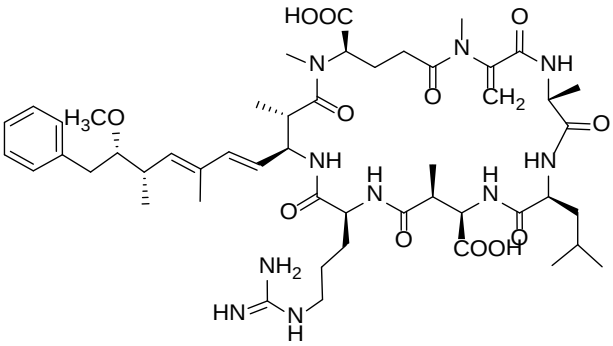
46 Prochlorosin 1.7



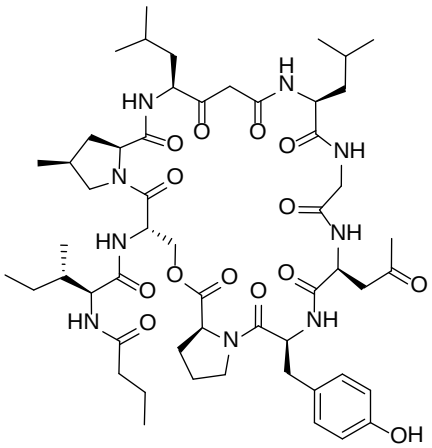
47 Hectochlorin



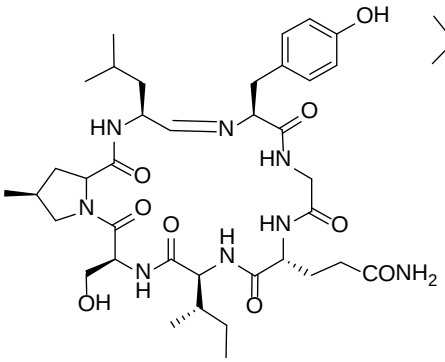
48 Anabaenopeptilide 90B



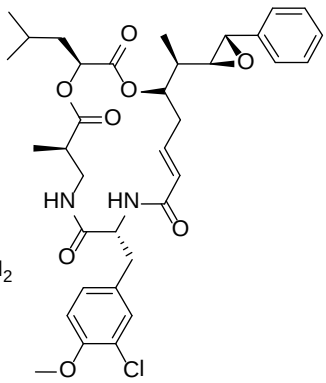
49 Microcystin-LR



Nostopeptolide A1
50



Nostocyclopeptolide A1
51



Cryptophycin 1
52

In cyanobacteria, peptides produced by two types of biosynthesis pathways: by ribosomal pathway with post-translational modifications or by non-ribosomal pathway often combined with polyketide pathway (mixed NRPS-PKS pathway) (Dittmann *et al.*, 2001; Kehr *et al.*, 2011). Microviridins (44) from *Microcystis* and *Planktothrix*, patellamides (45) from *Prochloron* spp. (originally from *Lissoclinum patella*), or prochlorosins (46) from *Prochlorococcus* MIT9313 are produced by the ribosomal pathway (Sivonen *et al.*, 2010). Anabaenopeptilide (48) produced by *Anabaena* strain 90 (Rouhiainen *et al.*, 2000), anabaenopeptins from strain *Planktothrix agardhii* CYA 126/8 (Christiansen *et al.*, 2011) were synthesized by only a single NRPS gene cluster (without PKS modules). Hectochlorin (47), a remarkable antifungal and cytotoxic compound from *Lyngbya majuscula* (Ramaswamy *et al.*, 2001) or the microcystins (49) from *Microcystis aeruginosa* (Tillett *et al.*, 2000); nodularin from *Nodularia spumigena* (Moffitt *et al.*, 2001; Moffitt *et al.*, 2004); nostopeptolide (50), nostocyclopeptide (51) and cryptophycin (52) from *Nostoc* spp.; or aeruginosin from *Microcystis aeruginosa* are produced via a mixed NRPS/PKS pathway (Ersmark *et al.*, 2008; Kehr *et al.*, 2011). The enormous diversity of cyanobacterial peptides, e.g. the cyclic structure and presence of unusual and modified amino acids, assumed that the majority of them are synthesized by the non-ribosomal synthetic pathway which including a large number of multidomain synthetases placed on the inner membrane of the cyanobacterial cell (Welker *et al.*, 2006; Kehr *et al.*, 2011). Because the position of the enzyme decides the final amino acid sequence, every change in function of a particular enzyme then results in the production of a different peptide variant. Despite the production of cyanobacterial peptides being widely studied from many aspects, the primary function of them still remains unresolved (Hrouzek, 2010; Chu, 2012).

There are different hypotheses involving in the function of cyanopeptides in the physiology and ecology of cyanobacteria. The compounds might function as protective compounds (Welker *et al.*,

2006), much as antiherbivores (Carmichael, 1992; Jones *et al.*, 1999) and allelopathic chemicals (Schagerl *et al.*, 2002; Suikkanen *et al.*, 2004; Dahms *et al.*, 2006; Shanab, 2007; Gantar *et al.*, 2008; Leão *et al.*, 2009) or signaling compounds between cells (Dittmann *et al.*, 2001; Ferreira, 2006). However, it is likely that we still know very little about these metabolites.

IV.4. Effect of culture conditions on growth and active compounds of Bio 33

Up to now, most of studies about the regulation of production of secondary metabolites in cyanobacteria focused on the hepatotoxic microcystins (Watanabe *et al.*, 1985; Watanabe *et al.*, 1989; Utkilen *et al.*, 1992; Vézie *et al.*, 2002; Tonk *et al.*, 2005), cylindrospermopsin (Dyble *et al.*, 2006; Cirés *et al.*, 2011), nodularins (Lehtimäki *et al.*, 1997), and the potent neurotoxic anatoxins (Rapala *et al.*, 1993). Besides, the influence of culture conditions on production of some promising antifungal and cytotoxic agents, including laxaphycin A and B and curacin A in *Lyngbya majuscula* were also taken into account (Burja *et al.*, 2002). Experimental factors such as temperature, light, nitrate, phosphorus, salinity and pH are commonly tested (Wiedner *et al.*, 2003; Mazur-Marzec *et al.*, 2005; Dyble *et al.*, 2006; Carneiro *et al.*, 2009; Kurmayer, 2011). Therefore, the understanding of how culture conditions affect the production of the antifungal lipopeptides in Bio 33 is very important and necessary to control and optimize the biosynthesis for further research. Nevertheless, the extracellular balticidins concentrations of strain Bio 33 were not identified because they remained too low for the measurement.

The distribution of cyanobacteria in the environment potentially reflects their different need and sensitivity regarding many factors such as temperature, radiation, salinity, and nutrients. In the summer of 1994 and 1997, a vertical distribution pattern of the cyanobacteria *Nodularia spumigena, Aphanizomenon* sp., and *Anabaena* spp. in the water column of the open Baltic Sea was analyzed (Hajdu *et al.*, 2007). These cyanobacteria are dominating species in late summer water blooms in the Baltic Sea (Lehtimäki *et al.*, 1997; Lehtimäki, 2000; Hajdu *et al.*, 2007; Sivonen *et al.*, 2007). It is reported that *Nodularia spumigena* accumulated mainly in the top 5 m of the water mass, while *Aphanizomenon* sp. was found in the whole water column (0 - 20 m) and had bimodal vertical distributions (Hajdu *et al.*, 2007). *Anabaena* sp. in the Baltic Sea is often found at about 2 - 8 m depth and also found down to 20 m in the sea water, and had bimodal abundance depth distributions with the deeper peak somewhat shallower than *Aphanizomenon* sp. (Hajdu *et al.*, 2007). According to Mohlin (2010) the vertical separation reflects the sensitivity to high radiation and photoprotective strategies. Therefore, it suggests that *Anabaena* sp. in the Baltic Sea cannot tolerate high light intensities and prefers lower temperature.

The ability of the cyanobacteria to produce antibiotic compounds could be an advantage for their survival in natural environment (Teuscher, 1992; Kreitlow *et al.*, 1999). In the other hand, changes of environmental conditions may result in entirely difference in biological activities (Schlegel *et al.*, 1998). In fact, changes in active compound production of cyanobacteria as a response to various environmental factors including temperature, salinity, radiation, and nutrient concentrations have been studied in laboratory (Rapala *et al.*, 1997; Panosso *et al.*, 2000; Repka *et al.*, 2001; Hobson *et al.*, 2003; Mazur-Marzec *et al.*, 2005). Thus, we studied the effects of culture conditions on the growth of strain Bio 33 and the content of balticidins with the aim to investigate the culture conditions for the optimum production of the active compounds.

In our experiments, the growth and the intracellular antifungal lipopeptide concentration of the Baltic Sea cyanobacterium *Anabaena* sp. strain Bio 33 was studied in batch cultures under different temperatures, light intensities, nitrate concentrations and cobalt supplements. The cyanobacterial growth was evaluated by estimation of the dry weight of biomass and the lipopeptide concentrations were detected by HPLC. Because the change in the area of the main peaks recorded under different cultivating conditions reflected the effect of these factors on the production of active compounds, these experiments allowed us to study the effects of growth conditions on the production of antifungal compounds in comparison to differences in growth rates. Method validation for quantification of the antifungal compounds was performed using the control biomass of Bio 33 cultivated in a 35 L aerated fermenter in BG 11 medium + 0.5% NaCl, pH 8.5, 22.5°C, light intensity 20 μmol photons m^{-2} s^{-1} under 24 h continuous irradiance. Without pure authentic standard, in our cultivation study, the detector responses obtained from each peak extracted from biomass cultivated under different conditions were compared to the detector response obtained from a certain amount of the control biomass. Normally, turbidity recorded as absorbance (or optical density (OD)) of cell cultures during growth is used to generate growth curves of unicellular microorganisms. However, because clumping phenomenon occurs during cultivation of filamentous cyanobacteria, measuring of dry weight is the most satisfactory method for these strain (Bui, 2006). Therefore, dry weight of biomasses of *Anabaena* sp. Bio 33 was estimated every 5 days.

IV.4.1. Effect of culture media

The mol N/P ratios are 100:1 and 20:1 for BG 11 and MBL (or the mass N/P ratios are 48:1 and 10:1, respectively). Comparison of biotest results and chemical nutrient concentrations in lakes has suggested that a mass N:P ratio above 17 indicates P limitation, a ratio below 10 indicates N limitation and values between 10 and 17 indicate that either of the nutrients may be limiting. It is generally accepted that high concentrations of P and a low N:P supply ratio promote the growth of cyanobacteria

and are favorable for the production of cyanobacteria blooms (Schindle, 1977; Niemi, 1979; Smith, 1983; Huebel, 1984; Pechar, 1992; Marinelli, 2009). However, Wasmund (1997) found that blooms occurred in the whole N:P ratio range from 0 to more than 30. Blooming of cyanobacteria in areas where the N:P ratio is below the Redfield ratio of 16 (Niemi, 1979) is due to the ability of nitrogen fixing. In the Baltic Sea, generally, the spring bloom of diatoms led to low N:P ratios in the surface water in summer (Sivonen *et al.*, 2007). Lately in summer, after the decline of diatoms, the bloom of cyanobacteria occurred (Sivonen *et al.*, 2007). Under these conditions, the N-fixing cyanobacteria are better competitors than other species of phytoplankton whose growth depends on dissolved nitrogen (Wasmund, 1997; Suikkanen, 2008). Wasmund also suggested that the low N:P ratio may be a prerequisite but not necessarily the trigger for the bloom because the observed N:P ratio was low long before the bloom occurred (Wasmund, 1997). In the other hand, nutrient concentrations are more relevant for bloom formation than the ratios (Vincent, 1989).

The nitrate concentration in BG 11 and MBL media are 1.5 and 0.085 g/L, respectively. The phosphate concentration in BG 11 and MBL media are 0.031 and 0.0087 g/L, respectively. It means the nitrate and phosphate concentration in BG 11 medium are about 18 and 4 times higher than in MBL medium, respectively. The BG 11 medium was more suitable for Bio 33 to overcome the physical and chemical adaption process than the low nutrient MBL medium. As a result, the strain produced more biomass in BG 11 medium than in MBL medium. In addition, the antifungal lipopeptide concentration of Bio 33 cultivated in BG 11 increased faster and reached the maximum concentration sooner (before day 20^{th}) in comparison to MBL medium (after day 20^{th}). Hence, although the antifungal lipopeptide concentration produced by Bio 33 cultivated in MBL was highest (at 22.5°C, day 30^{th}) and seemed to be still increasing, BG 11 was considered to be the better medium for the growth of Bio 33 and for the production of balticidins.

As balticidins are nitrogen-rich compounds with 11 mol nitrogen x mol lipopeptide^{-1} (Figure III-20), we expected lower balticidin concentrations in the biomass of Bio 33 cultivated in media without nitrate. However, in BG 11 and MBL media the concentration of balticidins was nearly identical in nitrate-free and nitrate-rich medium. In addition, nitrate had no effect on growth of Bio 33 in BG 11 and MBL media. The effect of nitrate on the production of balticidins was clear only in BG 11 medium at 22.5°C - when the production of balticidins was highest. At this temperature, the depletion of nitrate from BG 11 medium did not affect the production of balticidin A but increased the production of balticidin B and decreased the production of balticidin C. The phenomenon that nitrate had no effect on the production of balticidins by Bio 33 might be explained by the nitrogen-fixing capacity of the strain. It seems that the nitrogen fixation was sufficient to support the production of balticidins in these media.

Nevertheless, further research is needed to clarify the relationship among balticidins A, B, C as well as the biosynthesis of them.

IV.4.2. Effect of temperature

The growth of Bio 33 was lowest at 15°C and highest at 26°C, the production of balticidins was highest at 22.5°C and lowest at 30°C. These temperatures also belong to the common range temperature reported in many publications. Investigations of cyanotoxins found out, that intracellular toxins were usually highest under conditions which also favored growth (Sivonen, 1990). Normally, the optimum temperature for growth is also the optimum temperature for the production of bioactive compounds (Robarts *et al.*, 1987). Nevertheless, it was also published that optimum conditions for growth (pH, temperature, and light) did not coincide with those for toxin production by the *M. aeruginosa* cultures (Westhuizen *et al.*, 1985).

There are many reports about the correlation between temperature and the growth as well as the secondary metabolite concentration in cyanobacteria (Niemi, 1979; Huebel, 1984; Wasmund, 1997; Ferreira, 2006). Normally, the growth of cyanobacteria is directly affected by temperature and varies from species to species. Many species have been found to grow well in temperature range from 15 – 35°C (Table IV-3). The optimal temperatures for growth of some cyanobacteria are listed in Table IV-3. In the Baltic Sea, high biomass values of *Aphanizomenon* sp. and *Nodularia spumigena* occurred only in summer when water temperature was greater than 16°C (Niemi, 1979; Huebel, 1984; Wasmund, 1997). Besides, most cyanobacteria blooms occur during summer (Utkilen *et al.*, 1999). Generally, optimum temperatures for species of the genera *Microcystis*, *Anabaena*, *Aphanizomenon*, and *Oscillatoria* to gain maximum growth rate are above 20°C (Robarts *et al.*, 1987). It can be explained by the enhancement of nitrogen fixation rates and phosphorus remineralization with increasing water temperatures (Nordin *et al.*, 1980; Huebel, 1984; Robarts *et al.*, 1987; Wasmund, 1997).

The optimum temperatures for production of bioactive substances also vary from species to species. It was reported that optimum condition for anatoxin-a synthesis by toxic *Anabaena* sp. to was 22.5°C and a light intensity from 25 to 94 μmol photons m^{-2} s^{-1} (Collins, 1978). Reported optimal temperature for hepatotoxin production ranges from 18°C to 25°C depending on the strain used (van der Westhuizen *et al.*, 1983; Watanabe *et al.*, 1985; Sivonen, 1990; Rapala *et al.*, 1993). The maximum growth rates of many bloom-forming cyanobacteria are achieved at temperatures greater than 25°C (Ferreira, 2006). Temperature had the most significant effect on anatoxin-a concentration in the cells of *Anabaena* sp. (Rapala *et al.*, 1993). Anatoxin-a concentration was highest at 20°C and lowest at 30°C by all studied strains (Collins, 1978; Rapala *et al.*, 1993). Normally, the lowest production of

bioactive compounds was found at 30°C or higher (Sivonen, 1990). The same phenomenon was also observed with strain Bio 33 as the lowest amount of balticidins was produced at 30°C in the tested range of temperature from 15°C to 30°C.

Table IV-3. Optimal temperature for growth of some cyanobacteria

Species	$T_{optimal}$ (°C)	μ_{max} (day^{-1})	Reference
Microcystis sp.	28.8 – 30.5	0.25 – 0.30	(Krüger *et al.*, 1978)
Microcystis aeruginosa	27.5	0.8	(Nicklisch *et al.*, 1983)
Microcystis aeruginosa	32	0.59	(Watanabe *et al.*, 1985)
Microcystis aeruginosa	32	0.81	(Westhuizen *et al.*, 1985)
Microcystis sp.	30 – 35	-	(Imamura, 1981)
Anabaena oscillarioides	28	0.8	(Vincent *et al.*, 1979)
Anabaena spiroides	24	0.9	(Seki *et al.*, 1981)
Anabaena variabilis	35	1.1	(Collins *et al.*, 1982)
Anabaena sp.	30	-	(Novak *et al.*, 1985)
Anabaena sp.	25	-	(Imamura, 1981)
Anabaena flos-aquae	18	-	(Foy *et al.*, 1976)
Aphanizomenon flos-aquae	15	-	(Foy *et al.*, 1976)
Aphanizomenon flos-aquae	28	1.2	(Uehlinger, 1981)
Aphanizomenon sp.	25	-	(Imamura, 1981)
Microcystis aeruginosa	32	-	(Watanabe *et al.*, 1985)
Oscillatoria agardhii	23-25	0.59	(Ahlgren, 1978)
Oscillatoria agardhii	25	-	(Sivonen, 1990)
Oscillatoria agardhii	≥ 30	-	(Post *et al.*, 1985)
Oscillatoria sp.	27	-	(Novak *et al.*, 1985)
Oscillatoria agardhii	16		(Foy *et al.*, 1976)
Oscillatoria redekei	6	-	(Foy *et al.*, 1976)
Oscillatoria spp. *Phormidium* spp.	15-35	-	(Tang *et al.*, 1997)
Anabaena, *Aphanizomenon,* *Microcystis*	25 25 25	0.50 0.40 0.18	(Konopka *et al.*, 1978)

Adapted from Robarts *et al.* (1987).

It has been known from many studies that toxicity and intracellular toxins increased during the exponential phase, reached the highest concentration at the beginning of the stationary phase and then decreased (Devlin *et al.*, 1977; van der Westhuizen *et al.*, 1983; Watanabe *et al.*, 1989). In most experiments, our strain Bio 33 has not reached the stationary phase at the 30th day. A correlation between the biomass and the concentration of balticidins showed that, when the strain was cultivated at the optimum temperature for growth or at nearby temperatures, the concentration of balticidins increased since the beginning and reached the highest concentration in the exponential phase. At higher temperatures, the production of balticidins became slower. At 30°C, the production of balticidins had a reverse correlation with cultivation time.

The inhibition of growth due to high temperature can be explained by photorespiration, a process occurs when there is a high concentration of oxygen relative to carbon dioxide and correlates with temperature (Liere *et al.*, 1982). In this case, at the first step of the Calvin-Benson cycle, enzyme rubisco adds oxygen, instead of carbon dioxide as during normal photosynthesis, to ribulose-1,5-bisphosphate (RuBP). Because photorespiration is an ATP consuming process and leads to a net loss of carbon and nitrogen (as ammonia), it reduces the efficiency of photosynthesis and slows down the cyanobacterial growth (Liere *et al.*, 1982). Photorespiration has a major impact on cellular metabolism, particularly under high light intensity, high temperatures, and CO_2 or water deficits (Eisenhut *et al.*, 2008; Foyer *et al.*, 2009).

Cyanobacteria react to changes in light dose and temperature by varying their pigment contents, structural changes in cyanobacterial phycobilisomes, photosynthesis rates and growth rates (Foy *et al.*, 1982; Anderson *et al.*, 1983). In addition, the maximal photosynthetic activity was influenced by temperature through the rate of photosynthetic electron transfer (Gibson *et al.*, 1983; Post *et al.*, 1985). Photosynthetic electron transport is probably limiting at lower temperatures while transportation and fixation of carbon are probably limited at higher temperatures (Davison, 1991; Berges *et al.*, 2002).

IV.4.3. Effect of light intensity and period of light

Investigations in the effect of light received conflicting results. Some strains produced more active compounds under high light intensity but some preferred low light intensity. Sivonen (1990) suggested some reasons that could lead to conflicting results such as differences in the spectrum of the light sources, different behavior of strains and species, different culture media used, and/or differences in toxin detection methods.

In case of Bio 33, the strain grew better and produced more antifungal lipopeptides when light intensity was increased from 10 to 20 μmol photons m^{-2} s^{-1} and time of irradiance was increased from 12 h light to 24 h light. Higher temperatures led to a decreasing in growth or death. Undoubtedly, the effect of irradiance on growth and bioactive compound production by cyanobacteria depends on the species and strains studied. It was found that irradiance affected directly and was one of the most important factors that influenced the growth and the cyanotoxin production of cyanobacteria (Utkilen *et al.*, 1992; Rapala *et al.*, 1993; Wiedner *et al.*, 2003; Tonk *et al.*, 2005). Huber (1985) also found that among several environmental conditions tested, light was shown to be the most important factor affecting germination (Huber, 1985). 1999). Optimum light conditions could increase two fold the production of antibiotics (Chetsumon *et al.*, 1994). In most cases, when the growth of cyanobacteria was severely limited at low irradiances or inhibited at high irradiances, their intracellular toxin levels

were also low (Rapala *et al.*, 1993; Wiedner *et al.*, 2003). It is suggested that high temperature and high irradiances could increase the active exudation of cyanotoxins during natural blooms (Hobson *et al.*, 2003). Recently, increasing of 3-18% in fungicidal activity of some *Anabaena* spp. under continuous light at 40°C was recorded (Chaudhary *et al.*, 2012). Under continuous light, maximum growth rate was sufficiently maintained by a low but sustained rate of carbon input (Gibson *et al.*, 1983). Under these conditions, temperature was the main factor to limit the growth rate. Whereas, under light:dark cycle, the growth rate was limited by carbon fixation and the carbohydrate storage capacity (Gibson *et al.*, 1983). Maximum growth rate was only achieved when photosynthetic capacity was saturated (Gibson *et al.*, 1983; Robarts *et al.*, 1987). However, as mentioned by Gibson *et al.* (1983), because growth depends not only on the input of carbon during the light but also on the carbon losses during the dark period, a clear relationship between photosynthesis and growth is not necessary. Furthermore, the light quality was found to affect the bioactive compound synthetase (Kaebernick *et al.*, 2000; Mohlin, 2010). Therefore, in further studies, the impact of spectral composition of the artificial radiation in the laboratory should be investigated.

The optimum light intensity of 20 μmol photons m^{-2} s^{-1} identified for Bio 33 in our work was similar to the light intensity used for the Baltic Sea cyanobacteria cultivated in the work of Suikkanen *et al.* (2004). The sensitivity of strain Bio 33 to high irradiance could be deduced by the distribution of the *Anabaena* sp. in the Baltic Sea as this species distributes from 2 to 8 m and deep to 20 m under the water (Hajdu *et al.*, 2007). It suggests that the strain preferred low light intensity. Besides, the inhibition of photosynthesis at high irradiance caused by photooxidation could be an explanation for the sensitivity of Bio 33 to high irradiance. At high irradiance, photosynthetic rates are depressed by photooxidation and photorespiration (Liere *et al.*, 1982). The production of superoxide ion O_2^-, the principal agent of photooxidation, produced particularly at high concentration of oxygen and low concentration of CO_2, is promoted by high light irradiance. The superoxide ion is toxic and may cause damage to cytochromes, pigments and other components of the photosynthetic apparatus and thereby depresses subsequently the photosynthetic rate (Liere *et al.*, 1982).

IV.4.4. Effect of cobalt

There have been many studies about the effects of heavy metals on cyanobacteria (Saito *et al.*, 2002). Most of them focused on the toxic effect of heavy metal on cyanobacterial growth, N_2 fixation and photosynthesis (Rai *et al.*, 1989; Dubey *et al.*, 1990; El-Sheekh *et al.*, 2005; Balakrishnan *et al.*, 2007; Dudkowiak *et al.*, 2011). In form of coenzyme B_{12}, trace element cobalt plays an important role in many biological functions. Cobalt is required for the activity of several metaloenzymes such as methylmalonyl-CoA mutase, methionine synthase, and type II ribonucleotide reductase (Gleason *et*

al., 1976; 1980; Reichard, 1993; Kobayashi *et al.*, 1999; Raux *et al.*, 2000; Martens *et al.*, 2002; Nordlund *et al.*, 2006; Watanabe *et al.*, 2007; Bonnet *et al.*, 2010). Gleason *et al.* identified *nrdJ* gene and expressed the corresponding ribonucleotide reductase, an enzyme which is absolutely dependent on the addition of the cofactor, 5'-adenosylcobalamin, from *Anabaena* sp. strain PCC 7120 in *Escherichia coli* (Gleason *et al.*, 2002). It was also found that the *Anabaena* reductase showed over 90% sequence similarity to putative reductases found in genome sequences of other cyanobacteria, such as *Nostoc punctiforme*, *Synechococcus* sp. strain WH8102, and *Prochlorococcus marinus* MED4 (Gleason *et al.*, 2002). Some cyanobacteria can synthesize their own vitamin B_{12} and excrete it into culture medium with high level (Watanabe *et al.*, 2007; Bonnet *et al.*, 2010; Hashimoto *et al.*, 2012). A study of Bonnet *et al.* stated that the axenic N_2-fixing marine cyanobacterium *Crocosphaera watsonii* has B_{12} synthesis capacity of 1 x 10^{-18} (pM $cell^{-1}$ over exponential phase) (Bonnet *et al.*, 2010). Besides, Bonnet *et al.* (2010) also found that the axenic N_2-fixing marine cyanobacterium *Crocosphaera watsonii* excreted B_{12} up to 40 times higher per cell, compared to the smaller non–N_2-fixing strain of marine *Synechococcus*. The marine cyanobacteria *Prochlorococcus* and *Synechococcus bacillaris* absolutely required cobalt (Saito *et al.*, 2002; Leão *et al.*, 2007). Their production of Co binding ligands – referred as "cobalophores" – would increase the solubility of Co in seawater, protect the metal from particle scavenging, precipitation, and oxidation — thereby facilitating its bioavailability and uptake by cyanobacteria, and probably by other marine organisms (Saito *et al.*, 2002).

Besides, Howarth *et al.* (1988) concluded that nitrogen fixation is not limited by trace metals in the Baltic Sea (Howarth *et al.*, 1988). However, at concentration higher than 1 μM, cobalt was growth inhibitory for some cyanobacteria (Vla, 1980; Singh, 1989). The toxicity is due to inhibiting of active transport of K^+ and Na^+ ions into cyanobacterial cells by salts of heavy metals (Vla, 1980). The demand of trace element cobalt and its growth limiting level differ from species to species in cyanobacteria. Nevertheless, the concentration of cobalt in our study, 0.042 μM (in MBL medium) and 0.17 μM (in BG 11 medium with cobalt) was not high enough for inhibition the growth of Bio 33. Otherwise, the requirement for cobalt of Bio 33 seemed to be so low that only mild inhibiting effects on growth and biomass production of Bio 33 were observed at the lowest possible free cobalt concentration in BG 11 and MBL. In addition, this phenomenon was observed only at 22.5°C. It was assumed that there was a rapid recycling of cobalt in Bio 33 to avoid inducing cobalt limitation. Sunda *et al.* (1995) indicated that Co and Zn can replace one another metabolically in eukaryotic species. In addition, cobalt concentration and Co : Zn ratio could also be important. However, no report on the interreplacement of Co and Zn in cyanobacteria has been found so far. Therefore, further researches should be carried out to clarify the role of cobalt.

As a high cobalt concentration can inhibit the growth of cyanobacteria (VIa, 1980; Singh, 1989), it seems that cobalt can promote the lipopeptide production as a protector. Extracellular metabolites of *Anabaena doliolum* in a 1:1 ratio (v/v) with fresh culture medium could mediate the toxicity of heavy metal (Dubey *et al.*, 1990). Presumably, strain Bio 33 produced more balticidins as a regulation mechanism of toxicity caused by cobalt if the cobalt concentration in media is higher than necessary. Until now, little is known about the trace metal requirements of the cyanobacterium Bio 33. Thus, further studies are needed to elucidate the correlation among growth, balticidins biosynthesis and cobalt concentration.

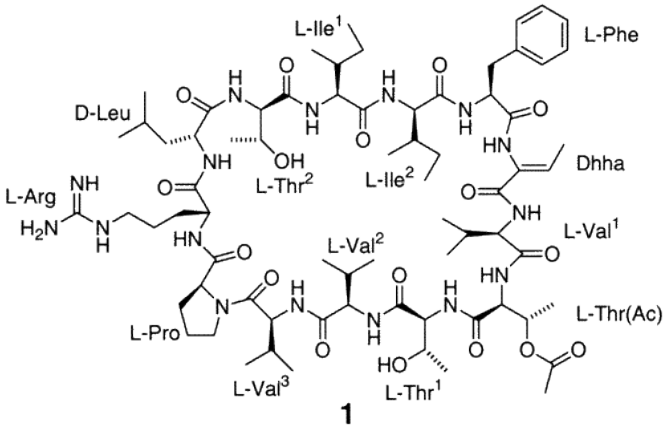
IV.5. Influence of cobalt and the strange round cells on the antimicrobial activity of strain TVN40

Strain TVN40 was first screened for antimicrobial activity in the work of Le (2010). A broad antimicrobial activity of the crude extract of the culture medium was found against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus* and *Candida maltosa*. No antimicrobial activity was found for the intracellular extracts of this strain (2 mg extract/paper disc) but antifungal and antibacterial activity was reported for the EtOAc extracts of strain TVN40. The bioassay-guided separation of the EtOAc extract of the culture medium led to the isolation of flourensadiol (57) ((1R,1aβ,4aα,7aα,7bβ)-Decahydro-4α-hydroxy-1,4,7β-trimethyl-1H-cycloprop[e]azulene-1-methanol; CAS 55812-89-0). This aromadendrane sesquiterpene was first isolated from the West Texas shrub *Flourensia cernua* (Kingston *et al.*, 1975; Pettersen *et al.*, 1975) and as far as known, the shrub *Flourensia cernua* is the only species from which flourensadiol was isolated. Crude extracts and fractions from this species have been demonstrated to have phytotoxic, antifungal, antialgal and antitermite activities, and reduce the consumption of alfalfa (*Medicago sativa* L.) pellets by sheep (Estell *et al.*, 1994; Tellez *et al.*, 2001; Martínez-Luis *et al.*, 2007; Estell *et al.*, 2008). Flourensadiol together with dehydroflourensic acid and methyl orsellinate described in the study of Mata (Mata *et al.*, 2003) are calmodulin (CaM) inhibitors and are the major phytotoxins of the crude extract of *Flourensia cernua* (Asteraceae) which inhibited radicle growth of *Amaranthus hypochondriacus* and *Echinochloa crus-galli*. Many other aromadendrane sesquiterpenoids which have similar structure with flourensadiol isolated from the leaves of *Xylopia brasiliensis* were reported exhibiting antifungal activity (Moreira *et al.*, 2003). Nevertheless, only the structure of flourensadiol was published, but little else is known about its biological activity.

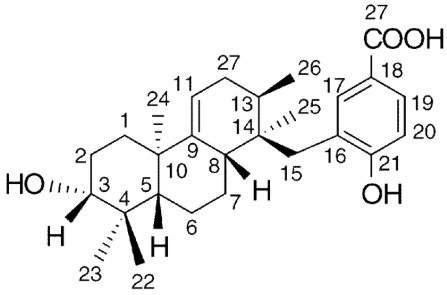
As far as known, flourensadiol as well as other antifungal aromadendrane sesquiterpenoid derivatives was found only in plant. These sesquiterpene derivatives are rare in cyanobacteria. So far, strain TVN40 is the first and only cyanobacterium from which flourensadiol was isolated. In Le's work, flourensadiol was isolated as an extracellular secondary metabolite of the culture medium of

cyanobacterium strain TVN40. In addition, the antimicrobial properties of different fractions of the culture medium suggested the presence of more than one active compound. Thus, the aim of our work was to develop a simpler and more effective method than the currently used EtOAc extraction to isolate active compounds from the culture medium of TVN40 and to identify some more active compounds besides of flourensadiol.

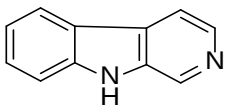
In aquatic environment, most of cyanobacterial metabolites, including cyanotoxins, are usually contained mainly within the cyanobacterial cells (Jones *et al.*, 1999; Mankiewicz *et al.*, 2003). Release of toxins from cells to the surrounding water appears during cell senescence, death and lysis, rather than by continuous excretion (Van Apeldoorn *et al.*, 2007). So far, only some exometabolites were identified as bioactive compounds, such as antibacterial diterpenoids noscomins A-E (**53**) from *Nostoc commune* (Jaki *et al.*, 1999; Jaki *et al.*, 2000), antifungal peptides tolybyssidins A-B (**54**) from *Tolypotrix byssoidea* (Jaki *et al.*, 2001), the algicidal indol alkaloid norharmane (**55**) from *Nodularia harveyana* (Volk *et al.*, 2006) or the algicidal phenolic compound 4,4'-dihydroxybiphenyl (**56**) from *Nostoc insulare* (Volk *et al.*, 2006). Excretion is enhanced by chemical treatments for the eradication of cyanobacteria, especially the use of algicides (either copper sulphate or organic herbicides) (Van Apeldoorn *et al.*, 2007). Nevertheless, the sesquiterpene flourensadiol (**57**) was found to be released to the surrounding culture media (Le, 2010). It was shown that, strain TVN40 produced and excreted flourensadiol to surrounding medium when it was cultivated in BG 11 medium supplemented with cobalt. This could be an evidence that there is a relationship between cobalt and the secondary metabolite production.



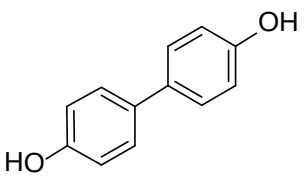
54 Tolybyssidin A (Jaki *et al.*, 2001)



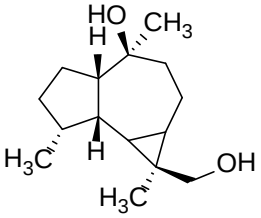
53 Noscomin A (Jaki *et al.*, 1999)



55 Norharmane



56 4,4'-dihydroxybiphenyl



57 Flourensadiol (Volk *et al.*, 2006)

To re-isolate flourensadiol from culture medium of cyanobacterium strain TVN40, a cultivation experiment was designed as well as an effective extraction method was developed using the resin XAD 16, binding organic substances with small to medium molecular weight from aqueous media (Heinek, 2011). Unfortunately, flourensadiol was not detectable in EtOAc extract by MS analysis of all culture media (before and after axenization). However, the antimicrobial assay confirmed that there was at least another compound which is responsible for the antifungal and antibacterial activity of the EtOAc extract and also of the fractions prepared by eluting the XAD-bounded substances with different solvents (Heinek, 2011).

At the same time, a microscopic analysis of the TVN40 culture revealed that the filaments grew together with strange round cells (SRC) which could have important effects on the growth as well as the active compound production of strain TVN40. The filamentous cyanobacterium strain TVN40 was successfully separated from the strange round cells and pure cultures of them were established. The antimicrobial activity testing of their pure and mixed culture extracts was done. As a result, only TVN40 was responsible for the antifungal activity but both TVN40 and SRC exhibited the antibacterial activity. Besides, the co-cultivation of SRCs with TVN40 reduced the antimicrobial activity of this strain.

Before the cultivation of pure TVN40 and SRC, whether flourensadiol was produced by TVN40 or SRC was unclear. However, the absence of flourensadiol in MS analysis of strain TVN40+SRC could due to the very low concentration of this compound in MS samples. Some other reasons that could be responsible for the absence of flourensadiol were also proposed. In the laboratory cultivation, the variation of some parameters which affect the involved secondary metabolism may result in the absence of the active products (Cannell *et al.*, 1988b). It explained why some cyanobacteria could no longer produce their active compounds after a long time cultivated under laboratory conditions. Another reason is that harvesting not at the right stage for production of antimicrobial compounds could led to an overlook of an undetectable concentration of active compounds (Cannell *et al.*, 1988b). A possible reason could be that accompanying heterotrophic bacteria in cyanobacterial culture might produce this compound and released it to the culture medium. According to Cole (1982), accompanying heterotrophic bacteria alter or metabolize algal allelochemicals, or release either inhibiting or stimulating substances. It is known that the role of bacteria may be an active metabolic one, possibly involving the recycling of algal extracellular products and thereby enhanced the growth of cyanobacteria (Fitzsimons *et al.*, 1984). Therefore, it must be clarified whether the observed activity of culture media from TVN40 and SRC was due to cyanobacteria only, or to the associated heterotrophic bacteria present in the cultures. Isolation and identification of the accompanying

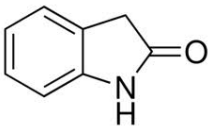
bacteria, their cultivation and testing their antimicrobial activity was a successful way. As result of the isolation process, 4 bacteria were isolated from the culture medium of SRC and were identified as *Devosia* sp., *Hydrogenophaga* sp., *Sphingomonas* sp., and *Rhodococcus* sp.. The mixture of the bacteria isolated from the culture medium of SRC was cultivated, extracted and the EtOAc extract was analyzed by HPLC/ESIMS. In the MS chromatograms of EtOAc extracts of non-axenic pure TVN40, SRC, and extracts from isolated bacteria of SRC, a fragment with *m/z* 239 corresponding to $[M+H]^+$ of flourensadiol was found. This signal was very high in the EtOAc extract of the culture medium of the mixture bacteria isolated from SRC but was low in other extracts. It suggested that the accompanying bacteria were the producer of flourensadiol. Therefore, further cultivation and extraction of each bacterium and MS analysis should be carried out to identify which bacterium is the producer of flourensadiol.

The extracellular compounds released from cyanobacteria can inhibit or stimulate the other cyanobacteria or microorganisms (Lopes *et al.*, 2011; Hashtroudi *et al.*, 2013). In case of cyanobacterial toxins, the release of them have been suggested to inhibit the same or other cyanobacterial species in the community, rather than inhibiting the abundance of competitors to cyanobacteria (Suikkanen *et al.*, 2004). Allelopathic effects of filamentous cyanobacteria in the Baltic Sea were studied by Sanna Suikkanen (Suikkanen *et al.*, 2004; Suikkanen *et al.*, 2006; Suikkanen, 2008) revealed that the filamentous cyanobacteria *Anabaena* sp., *Aphanizomenon flos-aquae* and *Nodularia spumigena* are capable of producing chemicals that inhibit competing phytoplankton species. The allelopathic effect of one cyanobacterium on the other varies from species to species. For example, the presence of *Microcystis aeruginosa* inhibited growth, reduced metabolic activity and an increased quantum requirement for biomass assimilation in green alga *Oocystis marsonii*, whereas no changes were observed in *Scenedesmus obliquus* (Dunker *et al.*, 2013). Recent investigations on fungicidal activity of extracellular filtrates from *Anabaena* sp. revealed that hydrolytic enzymes such as chitosanases, chitinases, may contribute to the fungicidal activity besides other bioactive compounds (Prasanna *et al.*, 2008; Gupta *et al.*, 2010; Prasanna *et al.*, 2010). Therefore, further experiments related to the co-cultivation of TVN40 filaments and SRC should be carried out to clarify the relationship between them. In addition, the agent which is responsible for the antifungal activity of TVN40 needs to be further explored.

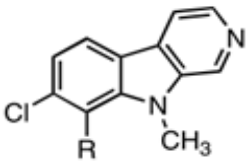
Many biologically active compounds and natural products possessing an oxindole framework with a hydroxy-bearing tetrasubstituted stereogenic center at C3 have been isolated from natural sources (Suárez-Castillo *et al.*, 2006). Particularly, some have been isolated from marine bryozoan, plant and bacteria (Tadera *et al.*, 1991; Balk-Bindseil *et al.*, 1995; Suárez-Castillo *et al.*, 2006). Oxindole (58)

derivatives and their structural relationship to indoles are important targets in medicinal and synthetic organic chemistry due to their broad biological activity including antifungal activity (Abele *et al.*, 2003; Porcs-Makkay *et al.*, 2004; Sharma *et al.*, 2010; Bergonzini *et al.*, 2012). Researches on secondary metabolites from higher plants frequently encountered indole alkaloids; they were also isolated, with a lesser extent, in microorganisms and animals (Gademann *et al.*, 2008). In contrast, there are much less cyanobacterial indole metabolites known so far (Gademann *et al.*, 2008). Among them were bauerines A-C (59-61) from the terrestrial cyanobacterium *Dichothrix baueriana* GO-25-5 (Larsen *et al.*, 1994), nostocarboline (62) from *Nostoc* 78-12A (Becher *et al.*, 2005), hapalindole A (63) from *Hapalosiphon fontinalis* (Moore *et al.*, 1987c), norharmane (55) from *Nodularia harveyana* (Volk, 2005; Volk *et al.*, 2006) (first identification of this compound from culture medium of cyanobacteria), the import plant hormone (phytohormane) auxin (64) or indole acetic acid from *Nostoc* and *Anabaena* spp. (Hashtroudi *et al.*, 2013). Some other cyanobacterial crude extracts were already reported for their auxin-like activity (as plant hormones) on potato tissue culture due to their containing indole derivatives (Shanab *et al.*, 2003). Welwitindolinone A isonitrile (65) and several related oxindole-containing alkaloids have been isolated from *Hapalosiphon welwischii* and *Westiella intricate* by Moore and co-workers (Stratmann *et al.*, 1994).

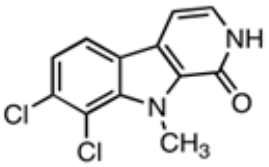
The dioxindole derivative (3-Hydroxy-2-oxo-2,3-dihydroindol-3-yl)acetonitrile (see III.6.5) was isolated from cabbage inoculated with *Pseudomonas cichorii* (Monde *et al.*, 1991). The compound could be derived via biological oxidation of indole-3-acetonitrile (Monde *et al.*, 1991). It was the first report of this dioxindole as a natural product. However, no report about the isolation of this dioxindole from cyanobacteria has been found so far. In our work, the dioxindole derivative was isolated from the culture medium of TVN40. Because dioxindole derivatives were mainly found in the presence of bacteria and the MS analysis (see III.6.10.2) displayed the presence of the fragment with *m/z* according to this dioxindole derivative in almost all extracts, it should be that the accompanying bacteria played an important role in the production of this compound. The HPLC/MS analysis of TYG medium also showed fragments with *m/z* 211 in positive mode and *m/z* 187 in negative mode. It is explained by the high tryptophane content of Pancreatic Digest of Casein which was used to prepare TYG medium. The ionization of this compound can lead to the presence of these fragments. Besides, the accompanying heterotrophic bacteria should be further investigated to figure out the producer of (3-Hydroxy-2-oxo-2,3-dihydroindol-3-yl)acetonitrile.



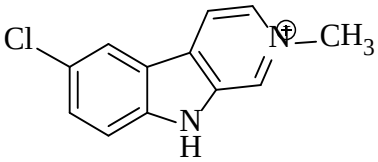
58 Oxindole



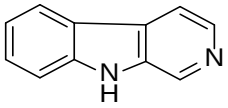
59 R = H: Bauerine A
60 R = Cl: Bauerine B



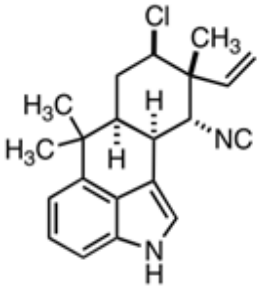
61 Bauerine C



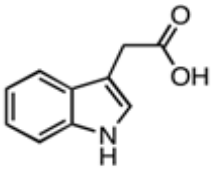
62 Nostocarboline



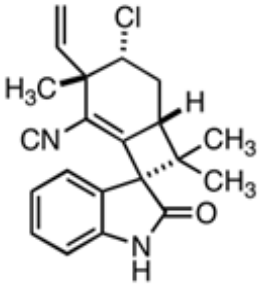
55 Norharmane



63..Hapalindole A



64 Auxin



65 Welwitindolinone A isonitrile

IV.6. Taxonomy

In our study, the 16S rRNA gene sequencing was applied to classify axenic cyanobacteria. 16S rRNAs are universal molecules, which evolved very early from the same ancestor and stayed quite constant during generations. Although secondary structure is highly conserved, the primary structure of 16S rRNA molecule, which is about 1500 nucleotides long, is a mosaic of conserved and variable regions. For this reason, in bacteria 16S rRNA gene sequence analysis is used to determine a broad range of relationships from phylum to species (Bryant, 1995).

For a correct taxonomic classification of strain Bio 33, the morphology was investigated and was in accordance with the current description of *Anabaena cylindrica*. This species shows straight and solitary unbranched trichomes, the vegetative cells are cylindrical, 3-4 μm wide and 3-5 μm long and the apical cells are rounded. The heterocysts are long and rounded, 5 μm wide and 6-8 μm long and intercalary in the filament (Geitler, 1932; Komárek *et al.*, 1989; Galhano *et al.*, 2011). Comparing the nucleotide sequence obtained from sequencing 16S rDNA of Bio 33 with sequence information available in the National Center for Biotechnology Information database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST) led to the sequence of *Anabaena cylindrica* as best hit (99%

max identity). In order to infer the phylogenetic position of our strain with the producer strains of the hassalidins, *Tolypothrix* and *Hassallia*, respectively, and other *Anabaena* spp., a phylogenetic tree (Figure III-68) was constructed on the basis of published partial 16S rRNA gene sequences of these genera using a maximum likelihood method. Basing on the taxonomic scheme of the cyanobacteria according to NCBI Taxonomy Browser (August 31, 2009) the order Nostocales are separated into four families Microchaetaceae (containing *Hassallia*, *Tolypothrix*), Nostocaceae (containing *Anabaena*), Rivulariaceae and Scytonemataceae. From the phylogenetic tree, it is clear that *Anabaena* belongs to a separate cluster compared to *Tolypothrix* and *Hassallia* spp.. It seems that *Tolypothrix* and *Hassallia* belong to a mixed cluster in the Microchaetaceae. It has already been indicated that *Tolypothrix* is a problematic taxon, as species of this genus are dispersed among other Microchaetacean genera such as *Spirirestis*, *Hassallia*, *Rexia*, and *Coleodesmium* (Vaccarino *et al.*, 2009). As provided by the authors, hassallidin A and B were isolated from an epilithic cyanobacterium identified as *Tolypothrix* species (basionym *Hassallia*) according to Geitler's characterization and taxonomy scheme (Neuhof *et al.*, 2005). However, no phylogenetic analysis of the *Hassallia* species has been published to date. From the molecular genetic data, it appears that our strain Bio 33 belongs to the *Anabaena* cluster in the family Nostocaceae and is completely distinguished from the published *Tolypothrix* and *Hassallia* spp., which means hassalidin-like lipopeptides are produced by different genera of the order Nostocales living under different environmental conditions.

In case of strain TVN40, early classification identified TVN40 as an *Anabaena* species basing on morphological features (Le, 2010). Nevertheless, the alignment of 16S rRNA gene sequence in our study shows that TNV40 belongs to the genus *Nostoc*. It confirmed that the classification basing on morphological characters may not reflect the true phylogenetic relationships of cyanobacteria.

Nostoc and *Anabaena* are traditionally separated based on morphological characteristics and life cycle differences (Henson *et al.*, 2002). Based on morphological features, the genera *Anabaena* and *Nostoc* belong to order Nostocales, family Nostocaceae by traditional classification (Anagnostidis *et al.*, 1989) and subsection IV.I by bacteriological classification (Rippka *et al.*, 2001). However, phylogenetic studies of cyanobacteria have demonstrated that genetic relationships sometimes conflict with the morphological classification because morphological characters can vary according to environmental conditions (Rajaniemi *et al.*, 2005). Formerly, *Nostoc* PCC 7120 was regarded as an *Anabaena* species, and *Nostoc* PCC 6720 was described as *Anabaenopsis* (Rippka *et al.*, 1979). In fact, the separation of the genera *Nostoc* and *Anabaena* into two distinct genera has also been discussed in recent years (Tamas *et al.*, 2000; Henson *et al.*, 2002; Rajaniemi *et al.*, 2005). 16S rRNA gene studies have shown that *Nostoc* and *Anabaena* are closely related; however, these studies were unable to

clearly differentiate between the two genera (Giovannoni *et al.*, 1988; Henson *et al.*, 2002). Based on partial (359 bp) *nifH* sequences, Tamas et al. suggested that genera *Nostoc* and *Anabaena* may belong within a single, broadly defined genus (Tamas *et al.*, 2000). However, alignment of the complete *nifD* sequences of Henson *et al.* (2002) suggests that *Nostoc* and *Anabaena* are distinct genera. Therefore, morphological, biochemical and molecular characterization need to be used to characterize these strains (Caudales *et al.*, 1992; Galhano *et al.*, 2011).

Until now, although the taxonomy of the cyanobacteria still remains to be answered, 16S rRNA gene sequences have proven to be very useful for the study of cyanobacterial phylogeny (Nelissen *et al.*, 1996). Recently, molecular approach has been employed to resolve evolutionary relationships within the cyanobacterial lineage using 16S rRNA associated with 23S rRNA; nitrogen fixation genes (*nif*HDK) with particular focus on *nifH* (Henson *et al.*, 2002); 16S rRNA with *rpoB* and, carbon-fixation-associated gene *rbcLX* (Rajaniemi *et al.*, 2005); DNA reassociation (Lachance, 1981), DNA polymorphisms within the phycocyanin locus (Neilan *et al.*, 1995). Nevertheless, even when molecular phylogenies have been employed to resolve evolutionary relationships within the cyanobacteria, the morphological chemotaxonomy and a combination of many candidates basing on molecular genetic approach are needed to classified cyanobacteria (Rajaniemi *et al.*, 2005; Komárek, 2010, 2011; Komárek *et al.*, 2012).

By 16S rRNA gene sequencing, SRC was identified as a single cell cyanobacterium species belonging to order Chroococcales. It is revealed that this microorganism is not another morphological form of strain TVN40.

V. Outlook

The lipopetides balticidins A-D, isolated from the biomass of *Anabaena cylindrica* strain Bio 33, displayed specific antifungal activity with only marginal cytotoxic effects on eukaryotic cells and belong to the hassallidin family. Temperature seems to have the most influence on growth of the strain in culture and on the production of balticidins. This study showed that, at the optimum temperature for the production of balticidins, the depletion of nitrate had no negative effect on the growth of Bio 33 but had different effects on the concentration of each balticidin. The effect of cobalt on growth and the production of balticidins differed according to temperature. Therefore, further research with different concentrations of nitrate and cobalt is needed to evaluate the relation between temperature and the concentration of nitrate as well as cobalt on the biosynthesis of balticidins.

Furthermore, the light quality was found to affect the bioactive compound synthetase (Kaebernick et al., 2000; Mohlin, 2010). Therefore, in further studies, the impact of spectral composition of the artificial radiation on the production of balticidins should be investigated.

Due to the specific antifungal activity of balticidins, further research is in progress to identify their antifungal mechanism basing on the ergosterol and glucan synthesis inhibition. First experiments with the main fraction FIII indicated a potential inhibition in the glucan synthesis. Therefore, the tests should be carried out with pure balticidins to clarify action mechanism.

In this study, we have shown a completely distinguished gene cluster between *Anabaena cylindrica* Bio 33 and *Hassallia* as well as *Tolypothrix* spp. available in the National Center for Biotechnology Information database using BLAST. Whether this separation is valid with the original strains producing hassallidins remains to be tested.

To date, limited information is available on the chemical nature of fungicidal metabolites in cyanobacteria and the gene(s) involved. Furthermore, identification of peptide synthetase genes would allow the detection of potential producers of hassalidin- and balticidin-like secondary metabolites in natural cyanobacterial populations as well as to reveal the role of these compounds to the producing organism and to the ecosystem. For these reasons, balticidin synthetase genes of Bio 33 should be identified to clarify the biosynthesis.

During this study, attempts to axenize the SRC were successful and according to the 16S rRNA gene the strains is identified belonging to order Chroococcales. In future, cultures of axenic SRC must be established, which is essential to study the characteristics of these microorganisms, and study their effects on the production of bioactive compounds from TVN40.

In addition, the agent which is responsible for the antifungal activity of TVN40 needs to be further explored. Furthermore, cultivation of each isolated bacterium from culture medium of SRC should be carried out. Cocultivation of isolated bacteria with SRC or TVN40 can be a promising way to research the antimicrobial activity under bacterial stress.

Summary of the thesis

Theme: "Cyanobacteria as source of new antifungals"

Represented by Thanh Huong Bui

During the last few decades, considerable side effects as well as numerous resistances against available antifungal agents promote the search for novel antifungal compounds. Meanwhile, hundreds of cyanobacterial secondary metabolites were reported. They exhibit a diverse spectrum of biological activities including antibacterial, algicidal, antifungal, antiviral, anticancer, cytotoxic, and enzyme inhibiting activities. Therefore, cyanobacteria are accepted as a source of potential pharmaceutical substances.

In the search for new antifungal agents, this study dealt with the antimicrobial screening, extraction, isolation, structural elucidation as well as selective biological investigations of the isolated compounds. In addition, the impact of the culture conditions on growth and on biosynthesis of bioactive compounds was also studied. Besides, selective cyanobacteria were axenized and the taxonomy as well as the genetic relationship of axenic cyanobacteria that produced bioactive compounds with some other cyanobacteria was identified basing on the 16S rRNA gene sequences.

22 Vietnamese and 6 German cyanobacterial strains were screened for their antifungal activity using the agar diffusion assay. Among them, the MeOH/water extract from the biomass obtained from a laboratory culture of strain Bio 33, isolated from the Baltic Sea near Rügen Island, exhibited a specific antifungal activity against *Candida maltosa*. Further investigation on human pathogenous fungi showed inhibition zones from 21 to 32 mm (1 mg/6 mm paper disc) to *Candida albicans*, *Candida krusei*, *Aspergillus fumigatus*, *Microsporum gypseum*, *Trichophyton rubrum* and *Mucor* sp. Besides, it was very impressed that extracts of strain Bio 33 showed no antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus.* The taxonomy basing on 16S rRNA gene sequence of the axenic Bio 33 identified this strain as *Anabaena cylindrica* species. As a result of the bioassay-guided fractionation of the crude MeOH/water extract, four new lipopeptides, named balticidins A – D, were isolated from the biomass of Bio 33. These lipopeptides represent a new structural type with the co-occurrence of a glycosylated cyclic peptide, a fatty acid containing chlorine and a disaccharide moiety. The main active fraction isolated from the MeOH/water extract of the biomass of Bio 33 which contains the four lipopeptides exhibited only marginal cytotoxic activity against the human bladder carcinoma cell line 5637 (IC_{50} = 93 μg/ml). The weak cytotoxic activity and the absence of antibacterial effects in the used *in vitro* test systems opens a promising

future for further investigations to clarify the antifungal mechanism and for *in vivo* applications of the new lipopeptides.

Different media, temperature, light intensity and period of irradiance - the most important factors that affect the growth and the production of bioactive compounds in cyanobacteria were investigated to figure out conditions at which Bio 33 produces maximum of balticidins under laboratory conditions. In addition, the depletion of nitrate and the trace element cobalt were studied to gain an insight into the production of balticidins. As a result, temperature was the most apparent factor influencing the growth of Bio 33 and the production of balticidins. Bio 33 was found to grow best in BG 11 medium plus 0.5% NaCl at 26°C, under white fluorescent continuous light and a light intensity of 20 μmol photons m^{-2} s^{-1}. Nevertheless, under the same conditions, 22.5°C was the best temperature for the production of balticidins. These experiments also indicated that harvesting of Bio 33 during the logarithmic growth phase, particularly at 20th day, should supply approximately maximum quantity of balticidins. At 22.5°C and 20 μmol photons m^{-2} s^{-1} under 24 h continuous irradiance, the depletion of nitrate had no negative effect on the growth and the concentration of balticidin A but increased balticidin B and decreased balticidin C; the absence of cobalt slightly decreased the growth but had no clear effect on the production of balticidins.

On the other hand, extracts of the culture medium of the Vietnamese cyanobacterium TVN40, exhibited antifungal activity against *Candida maltosa* and weak antibacterial activity. Extraction of the culture medium with XAD-16 and elution of the XAD-bounded compounds by different solvents resulted in five fractions (water, 80% MeOH, 100% MeOH, acetone, dichloromethan). The best activity was exhibited by the 80% MeOH fraction, so that this fraction was used for further isolation procedures. Four compounds have been isolated and one was identified as a dioxindole derivative. Structural elucidation of the other three compounds is still in progress.

TVN40 was formerly identified as an *Anabaena* sp. according to the morphological properties, but the 16S rRNA gene sequence confirms that the strain belongs to the genus *Nostoc*. Microscopic examination of TVN40 revealed that the filamentous strain was not a unialgal but a mixed culture with strange round cells (SRCs) - a unicellular cyanobacterium belonging to the order Chroococcales. Laboratory cultures of the pure filamentous strain TVN40, the isolated SRCs and the mixed culture of both strains were established. Both TVN40 and SRC culture media were responsible for the antibacterial activity against *B. subtilis*, *S. aureus* and *E. coli*. However, only the extract of the culture medium of TVN40 was active against *C. maltosa*. The supplement of cobalt enhanced the antimicrobial activity of the culture medium. Pure strains showed higher activity in comparison to the mixed culture of TVN40 and SRC.

VI. References

Abed, R. M. M.; Dobretsov, S. and Sudesh, K. (2009). Applications of cyanobacteria in biotechnology. *Journal of Applied Microbiology*. 106(1): 1-12.

Abele, E.; Abele, R.; Dzenitis, O. and Lukevics, E. (2003). Indole and isatin oximes: Synthesis, reactions, and biological activity. (review). *Chemistry of Heterocyclic Compounds*. 39(1): 3-35.

Ahlgren, G. (1978). Growth of *Oscillatoria agardhii* in chemostat culture. 2. Dependence of growth constants on temperature. . *Mitteilungen der internationalen Vereinigung für theoretische und angewandle Limnologie*. 21: 88-102.

Altschul, F. S.; Madden, L. T.; Schäffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W. and Lipman, J. D. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*. 25(17): 3389-3402.

Anagnostidis, K. and Komárek, J. (1985). Modern approach to the classification system of cyanophytes. 1 - Introduction. *Algological Studies/Archiv für Hydrobiologie, Supplement Volumes*. 38-39: 291-302.

(1986). Modern approach to the classification system of cyanophytes. 2. Chroococcales. *Algological Studies/Archiv für Hydrobiologie, Supplement Volumes*. 43: 157-226.

(1988). Modern approach to the classification system of cyanophytes. 3 - Oscillatoriales. *Algological Studies/Archiv für Hydrobiologie, Supplement Volumes*. 50-53: 327-472.

(1989). Modern approach to the classification system of Cyanophytes 4 - Nostocales. *Algological Studies/Archiv für Hydrobiologie, Supplement Volumes*. 56: 247-345.

(1990). Modern approach to the classification system of Cyanophytes. 5 - Stigonematales. *Algological Studies/Archiv für Hydrobiologie, Supplement Volumes*. 59: 1-73.

Andersen, R. A. (2005). Algal Culturing Techniques. *Elsevier Academic Press*.

Anderson, L. K.; Rayner, M. C.; Sweet, R. M. and Eiserling, F. A. (1983). Regulation of *Nostoc* sp. phycobilisome structure by light and temperature. *Journal of Bacteriology*. 155(3): 1407-1416.

Andriole, V. T. (1999). Current and future antifungal therapy: New targets for antifungal agents. *Journal of Antimicrobial Chemotherapy*. 44(2): 151-162.

Ansede, H. J.; Friedman, R. and Yoch, C. D. (2001). Phylogenetic analysis of culturable dimethyl sulfide-producing bacteria from a spartina-dominated salt marsh and estuarine water. *Applied and Environmental Microbiology*. 67(3): 1210-1217.

Aperis, G. and Mylonakis, E. (2006). Newer triazole antifungal agents: pharmacology, spectrum, clinical efficacy and limitations. *Expert Opinion on Investigational Drugs*. 15(6): 579-602.

Aráoz, R.; Molgó, J. and de Marsac, N. T. (2010). Neurotoxic cyanobacterial toxins. *Toxicon*. 56(5): 813-828.

Ashley, E. S. D.; Lewis, R.; Lewis, J. S.; Martin, C. and Andes, D. (2006). Pharmacology of systemic antifungal agents. *Clinical Infectious Diseases*. 43(Supplement 1): S28-S39.

Balakrishnan, C. P. and Narayanan, C. S. (2007). Phytotoxicity of heavy metals nickel and lead on the cyanobacterial pigments. *Seaweed Research and Utilisation*. 29: 1-2.

Balk-Bindseil, W.; Helmke, E.; Weyland, H. and Laatsch, H. (1995). Marine bacteria, VIII. Maremycin A and B, new diketopiperazines from a marine *Streptomyces* sp. *Liebigs Annalen*. 1995(7): 1291-1294.

Barker, L. A. G.; Hayes, P. K.; O'Mahony, S. L.; Vacharapiyasophon, P. and Walsby, A. E. (1999). A molecular and phenotypic analysis of *Nodularia* (Cyanobacteria) from the Baltic Sea. *Journal of Phycology*. 35: 931-937.

Barrett-Bee, K. J.; Lane, A. C. and Turner, R. W. (1986). The mode of antifungal action of tolnaftate. *Journal of Medical and Veterinary Mycology*. 24(2): 155-160.

Barrett, D. (2002). From natural products to clinically useful antifungals. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*. 1587(2–3): 224-233.

Becher, P. G.; Beuchat, J.; Gademann, K. and Jüttner, F. (2005). Nostocarboline: Isolation and synthesis of a new cholinesterase inhibitor from *Nostoc* 78-12A. *Journal of Natural Products*. 68(12): 1793-1795.

Belay, A. (2002). The potential application of *Spirulina* (*Arthrospira*) as a nutritional and therapeutic supplement in health management. *JANA-The Journal of the American Nutraceutical Association*. 5(2).

Beltran, E. C. and Neilan, A. B. (2000). Geographical segregation of the neurotoxin-producing cyanobacterium *Anabaena circinalis*. *Applied and Environmental Microbiology*. 66(10): 4468-4474.

Berges, J. A.; Varela, D. E. and Harrison, P. J. (2002). Effects of temperature on growth rate, cell composition and nitrogen metabolism in the marine diatom *Thalassiosira pseudonana* (Bacillariophyceae). *Marine Ecology Progress Series*. 225: 139-146.

Bergonzini, G. and Melchiorre, P. (2012). Dioxindole in asymmetric catalytic synthesis: Routes to enantioenriched 3-substituted 3-hydroxyoxindoles and the preparation of Maremycin A. *Angewandte Chemie*. 124(4): 995-998.

Bhadury, P. and Wright, P. C. (2004). Exploitation of marine algae: biogenic compounds for potential antifouling applications. *Planta*. 219(4): 561-578.

Bonjouklian, R.; Smitka, A. T.; Doolin, E. L.; Molloy, R. M.; Debono, M.; Shaffer, A. S.; Moore, E. R.; Stewart, B. J. and Patterson, M. L. G. (1991). Tjipanazoles, new antifungal agents from the blue-green alga *Tolypothrix tjipanasensis*. *Tetrahedron*. 47(37): 7739-7750.

Bonnet, S.; Webb, A. E.; Panzeca, C.; Karl, M. D.; Capone, G. D. and Sanudo-Wilhelmy, S. A. (2010). Vitamin B_{12} excretion by cultures of the marine cyanobacteria *Crocosphaera* and *Synechococcus*. *Limnology and Oceanography*. 55(5): 1959-1964.

Borelli, C.; Schaller, M.; Niewerth, M.; Nocker, K.; Baasner, B.; Berg, D.; Tiemann, R.; Tietjen, K.; Fugmann, B.; Lang-Fugmann, S. and Korting, H. C. (2008). Modes of action of the new arylguanidine abafungin beyond interference with ergosterol biosynthesis and in vitro activity against medically important fungi. *Chemotherapy*. 54(4): 245-259.

Borowitzka, M. A. (1995). Microalgae as sources of pharmaceuticals and other biologically active compounds. *Journal of Applied Phycology*. 7: 3-15.

Bossche, V. H.; Marichal, P. O. and Frank, C. (1994). Molecular mechanisms of drug resistance in fungi. *Trends in Microbiology*. 2(10): 393-400.

Bossche, V. H.; Engelen, M. and Rochette, F. (2003). Antifungal agents of use in animal health – chemical, biochemical and pharmacological aspects. *Journal of Veterinary Pharmacology and Therapeutics*. 26(5-29).

Bowman, M. S. and Free, J. S. (2006). The structure and synthesis of the fungal cell wall. *BioEssays*. 28: 799-808.

Brajtburg, J.; Powderly, W. G.; Kobayashi, G. S. and Medoff, G. (1990). Amphotericin B: current understanding of mechanisms of action. *Antimicrobial Agents and Chemotherapy*. 34(2): 183-188.

Bryant, D. A. (1995). The molecular biology of cyanobacteria. Dordrecht/Boston/London, *Kluwer Academic Publishers*.

Bucar, F.; Wube, A. and Schmid, M. (2013). Natural product isolation - how to get from biological material to pure compounds. *Natural Product Reports*. 30(4): 525-545.

Bueno, J. M.; Cuevas, J. C.; Fiandor, J. **M.; García**-Ochoa, S. and Gómez de las Heras, F. (2002). Antifungal sordarins. Synthesis and structure–activity relationships of 3',4'-Fused dioxolane and dioxane derivatives. *Bioorganic & Medicinal Chemistry Letters*. 12(2): 121-124.

Bui, H. T. N. (2006). Isolation of antibiotic and cytotoxic compounds from some Vietnamese cyanobacteria. *EMAU Greifswald*. Dissertation.

Burja, A. M.; Banaigs, B.; Abou-Mansour, E.; Grant Burgess, J. and Wright, P. C. (2001). Marine cyanobacteria -a prolific source of natural products. *Tetrahedron*. 57(46): 9347-9377.

Burja, A. M.; Abou-Mansour, E.; Banaigs, B.; Payri, C.; Burgess, J. G. and Wright, P. C. (2002). Culture of the marine cyanobacterium, *Lyngbya majuscula* (Oscillatoriaceae), for bioprocess intensified production of cyclic and linear lipopeptides. *Journal of Microbiological Methods*. 48: 207-219.

Cannell, J. P. R.; Kellam, S. J.; Owsianka, A. M. and Walker, J. M. (1988a). Results of a large scale screen of microalgae for the production of protease inhibitors. *Planta Medica*. 54(1): 10-14.

Cannell, J. P. R.; Owsianka, A. M. and Walker, J. M. (1988b). Results of a large-scale screening programme to detect antibacterial activity from freshwater algae. *British Phycological Journal*. 23(1): 41-44.

Cano, M.; Mulé, M.; Caire, G. and Halperin, D. (1990). Inhibition of *Candida albicans* and *Staphylococcus aureus* by phenolic compounds from the terrestrial cyanobacterium *Nostoc muscorum*. *Journal of Applied Phycology*. 2(1): 79-81.

Cappelletty, D. and Eiselstein-McKitrick, K. (2007). The Echinocandins. *Pharmacotherapy*. 27(3): 369-388.

Cardellina II, J. H.; Kirkup, M. P.; Moore, R. E.; Mynderse, J. S.; Seff, K. and Simmons, C. J. (1979). Hyellazone and chlorohyellazole, two novel carbazoles from the blue-green alga *Hyella caespitosa* born. et flah. *Tetrahedron Letters*. 20(51): 4915-4916.

Carmeli, S.; Moore, R. E. and Patterson, G. M. L. (1990a). Tolytoxin and new scytophycins from three species of *Scytonema*. *Journal of Natural Products*. 53(6): 1533-1542.

Carmeli, S.; Moore, R. E.; Patterson, G. M. L.; Mori, Y. and Suzuki, M. (1990b). Isonitriles from the blue-green alga *Scytonema mirabile*. *The Journal of Organic Chemistry*. 55(14): 4431-4438.

Carmichael, W. W.; Biggs, D. F. and Gorham, P. R. (1975). Toxicology and pharmacological action of *Anabaena flos-aquae* toxin. *Science*. 187(4176): 542-544.

Carmichael, W. W.; Mahmood, N. A. and Hyde, E. G. (1990). Natural toxins from cyanobacteria (blue-green algae). Marine Toxins. *American Chemical Society*. 418: 87-106.

Carmichael, W. W. (1992). Cyanobacteria secondary metabolites- the cyanotoxins. *Journal of Applied Bacteriology*. 72(6): 445-459.

Carneiro, R. L.; dos Santos, M. E. V.; Pacheco, A. B. F. and Azevedo, S. M. F. d. O. e. (2009). Effects of light intensity and light quality on growth and circadian rhythm of saxitoxins production in *Cylindrospermopsis raciborskii* (Cyanobacteria). *Journal of Plankton Research*. 31(5): 481-488.

Castenholz, R. W. (1989a). Order Stigonematales. Bergey's manual of systematic bacteriology. Staley J. T., Bryant M. P., Pfennig N. and Holt J. G. Baltimore: *Williams and Wilkins*. 3: 1794–1799.

(1989b). Order Oscillatoriales. Bergey's manual of systematic bacteriology. Staley J. T., Bryant M. P., Pfennig N. and Holt J. G. Baltimore: *Williams and Wilkins*. 3: 1771–1780.

Caudales, R. and Wells, J. M. (1992). Differentiation of free-living *Anabaena* and *Nostoc* cyanobacteria on the basis of fatty acid composition. *International Journal of Systematic Bacteriology*. 42(2): 246-251.

Chaganty, S.; Golakoti, T.; Heltzel, C.; Moore, R. E. and Yoshida, W. Y. (2004). Isolation and structure determination of cryptophycins 38, 326, and 327 from the terrestrial cyanobacterium *Nostoc* sp. GSV 224. *Journal of Natural Products*. 67(8): 1403-1406.

Chandran, S. and Singh, R. S. P. (2007). Comparison of various international guidelines for analytical method validation. *Die Pharmazie - An International Journal of Pharmaceutical Sciences*. 62(1): 4-11.

Chaplin, M. F. (1982). A rapid and sensitive method for the analysis of carbohydrate components in glycoproteins using gas-liquid chromatography. *Analytical Biochemistry*. 123(2): 336-341.

Chaudhary, V.; Prasanna, R. and Bhatnagar, A. K. (2012). Modulation of fungicidal potential of *Anabaena* strains by light and temperature. *Folia Microbiologica*. 57(3): 199-208.

Chen, S. C.-A.; Slavin, M. A. and Sorrell, T. C. (2011). Echinocandin antifungal drugs in fungal infections: A comparison. *Drugs*. 71(1): 11-41

Chetsumon, A.; Maeda, I.; Umeda, F.; Yagi, K.; Miura, Y. and Mizoguchi, T. (1994). Antibiotic production by the immobilized cyanobacterium, *Scytonema* sp. TISTR 8208, in a seaweed-type photobioreactor. *Journal of Applied Phycology*. 6(5-6): 539-543.

Christiansen, G.; Philmus, B.; Hemscheidt, T. and Kurmayer, R. (2011). Genetic variation of adenylation domains of the anabaenopeptin synthesis operon and evolution of substrate promiscuity. *Journal of Bacteriology*. 193(15): 3822-3831.

Chu, W.-L. (2012). Biotechnological applications of microalgae. *International e-Journal of Science, Medicine & Education*. 6(1): 24-37.

Chuang, P.-H.; Hsieh, P.-W.; Yang, Y.-L.; Hua, K.-F.; Chang, F.-R.; Shiea, J.; Wu, S.-H. and Wu, Y.-C. (2008). Cyclopeptides with anti-inflammatory activity from seeds of *Annona montana*. *Journal of Natural Products*. 71(8): 1365-1370.

Cirés, S.; Wörmer, L.; Timón, J.; Wiedner, C. and Quesada, A. (2011). Cylindrospermopsin production and release by the potentially invasive cyanobacterium Aphanizomenon ovalisporum under temperature and light gradients. *Harmful Algae*. 10(6): 668-675.

Cole, J. J. (1982). Interactions between bacteria and algae in aquatic ecosystems. *Annual Review of Ecology and Systematics*. 13(1): 291-314.

Collins, C. D. and Boylen, C. W. (1982). Physiological responses of *Anabaena variabilis* (cyanophyceae) to instantaneous exposure to various combinations of light intensity and temperature. *Journal of Phycology*. 18(2): 206-211.

Collins, M. (1978). Algal toxins. *Microbiological Reviews*. 42(4): 725-746.

Colyer, C. L.; Kinkade, C. S.; Viskari, P. J. and Landers, J. P. (2005). Analysis of cyanobacterial pigments and proteins by electrophoretic and chromatographic methods. *Analytical and Bioanalytical Chemistry*. 382(3): 559-569.

Congestri, R.; Federici, R. and Albertano, P. (2000). Evaluating biomass of Baltic filamentous cyanobacteria by image analysis. *Aquatic Microbial Ecology*. 22: 283–290.

D'Agostino, G.; J., d.-C.; Mellado, B.; Izquierdo, M. A.; Minarik, T.; Cirri, L.; Marini, L.; Perez-Gracia, J. L. and Scambia, G. (2006). A multi-center phase II study of the cryptophycin analogue LY355703 in patients with platinum-resistant ovarian cancer. *International Journal of Gynecological Cancer*. 16: 71-76.

Dahms, H.-U.; Ying, X. and Pfeiffer, C. (2006). Antifouling potential of cyanobacteria: a mini-review. *Biofouling*. 22(5): 317-327.

Dale, G. N.; Nurun, N. S. G.; Kevin, K. S.; Chowdhury, F. H.; Stanikunaite, R.; Mark, T. H. and Rajbandari, I. (2003). Secondary metabolites from plants and marine organisms as selective anti-cyanobacterial agents. Off-Flavors in Aquaculture. *ACS Publications*. 848: 179-194.

Davison, I. R. (1991). Environmental effects on algal photosynthesis: Temperature. *Journal of Phycology*. 27(1): 2-8.

Denning, D. W. (1997). Echinocandins and pneumocandins-a new antifungal class with a novel mode of action. *Journal of Antimicrobial Chemotherapy*. 40(5): 611-614.

Devlin, J. P.; Edwards, O. E.; Gorham, P. R.; Hunter, N. R.; Pike, R. K. and Stavric, B. (1977). Anatoxin-a, a toxic alkaloid from *Anabaena flos-aquae* NRC-44h. *Canadian Journal of Chemistry*. 55(8): 1367-1371.

Dieckmann, R.; Neuhof, T.; Preussel, K.; Schmieder, P.; Seibold, M. and von Döhren, H. (2006). Lipopeptides having pharmaceutical activity. EP1698638 A1.

Dittmann, E.; Neilan, B. A. and Börner, T. (2001). Molecular biology of peptide and polyketide biosynthesis in cyanobacteria. *Applied Microbiology and Biotechnology*. 57(4): 467-473.

Dixon, D. M. and Walsh, T. J. (1996). Antifungal agents. Medical Microbiology. Baron S. Galveston (TX): *University of Texas Medical Branch at Galveston*.

Dubey, S. K. and Rai, L. C. (1990). Heavy metal toxicity in a N_2-fixing cyanobacterium, *Anabaena doliolum*: regulation of toxicity by certain environmental factors. *Biomed. Environ. Sci.* . 3(2): 240-249.

Dudkowiak, A.; Olejarz, B.; Łukasiewicz, J.; Banaszek, J.; Sikora, J. and Wiktorowicz, K. (2011). Heavy metals effect on cyanobacteria *Synechocystis aquatilis* study using absorption, fluorescence, flow cytometry, and photothermal measurements. *International Journal of Thermophysics*. 32(4): 762-773.

Dunker, S.; Jakob, T. and Wilhelm, C. (2013). Contrasting effects of the cyanobacterium *Microcystis aeruginosa* on the growth and physiology of two green algae, *Oocystis marsonii* and *Scenedesmus obliquus*, revealed by flow cytometry. *Freshwater Biology*. 58(8): 1573-1587.

Dvorak, Z. (2011). Drug–drug interactions by azole antifungals: Beyond a dogma of CYP3A4 enzyme activity inhibition. *Toxicology Letters*. 202(2): 129-132.

Dyble, J.; Tester, P. A. and Litaker, R. W. (2006). Effects of light intensity on cylindrospermopsin production in the cyanobacterial HAB species *Cylindrospermopsis raciborskii*. *African Journal of Marine Science*. 28(2): 309-312.

Ebbinghaus, S.; Hersh, E.; Cunningham, C. C.; McDermott, S. O. D., D. ; Stephenson, J.; Richards, D. A.; Eckardt, J.; Haider, O. L. and Hammond, L. A. (2004). Phase II study of synthadotin (SYN-D; ILX651) administered daily for 5 consecutive days once every 3 weeks (qdx5q3w) in patients (Pts) with inoperable locally advanced or metastatic melanoma. *Journal of Clinical Oncology, 2004 ASCO Annual Meeting Proceedings (Post-Meeting Edition)* 22(14S).

Edwards, D. J.; Marquez, B. L.; Nogle, L. M.; McPhail, K.; Goeger, D. E.; Roberts, M. A. and Gerwick, W. H. (2004). Structure and biosynthesis of the Jamaicamides, new mixed polyketide-peptide neurotoxins from the marine cyanobacterium *Lyngbya majuscula*. *Chemistry & Biology*. 11(6): 817-833.

Eisenhut, M.; Ruth, W.; Haimovich, M.; Bauwe, H.; Kaplan, A. and Hagemann, M. (2008). The photorespiratory glycolate metabolism is essential for cyanobacteria and might have been conveyed endosymbiontically to plants. *Proceedings of the National Academy of Sciences*. 105(44): 17199-17204.

El-Sheekh, M. M.; El-Shouny, W. A.; Osman, M. E. H. and El-Gammal, E. W. E. (2005). Growth and heavy metals removal efficiency of *Nostoc muscorum* and *Anabaena subcylindrica* in sewage and industrial wastewater effluents. *Environmental Toxicology and Pharmacology*. 19(2): 357-365.

El Omari, M. (2011). Ambigols A-C and tjipanazole D: Bioinformatic analysis of their putative biosynthetic gene clusters Bonn, *Rheinischen Friedrich-Wilhelms-Universität Bonn*. Dissertation.

Ersmark, K.; Del Valle, J. R. and Hanessian, S. (2008). Chemistry and biology of the aeruginosin family of serine protease inhibitors. *Angewandte Chemie International Edition*. 47(7): 1202-1223.

Estell, R. E.; Anderson, D. M. and Havstad, K. M. (1994). Effects of organic solvents on use of tarbush by sheep. *Journal of Chemical Ecology*. 20(5): 1137-1142.

Estell, R. E.; Fredrickson, E. L.; Anderson, D. M. and Remmenga, M. D. (2008). Effects of cis-β-ocimene, cis-sabinene hydrate, and monoterpene and sesquiterpene mixtures on alfalfa pellet intake by lambs. *Journal of Animal Science*. 86(6): 1478-1484.

Felder, S.; Grabe, K. and Mayrhofer, T. (2010). Micafungin im Vergleich zu Caspofungin bei der Behandlung von invasiven Candida-Infektionen: Eine Kosteneffektivitätsanalyse für Deutschland. *PharmacoEconomics German Research Articles*. 8(2): 71-83.

Ferreira, A. H. F. (2006). Peptides in cyanobacteria under different environmental conditions *Technischen Universität Berlin*. Dissertation

Finni, T.; Kononen, K.; Olsonen, R. and Wallström, K. (2001). The history of cyanobacterial blooms in the Baltic Sea. *AMBIO: A Journal of the Human Environment*. 30(4): 172-178.

Fitzsimons, A. G. and Smith, R. V. (1984). The isolation and growth of axenic cultures of planktonic blue-green algae *British Phycological Journal*. 19: 157-162.

Fogg, G. E. and Thake, B. (1987). Chapter 2. Cultures of limited volume. Algal cultures and phytoplankton ecology. England: *The university of Winsconsin Press, Ltd.*

Foy, R. H.; Gibson, C. E. and Smith, R. V. (1976). The influence of daylength, light intensity and temperature on the growth rates of planktonic blue-green algae. *British Phycological Journal*. 11(2): 151-163.

Foy, R. H. and Gibson, C. E. (1982). Photosynthetic characteristics of planktonic blue-green algae: The response of twenty strains grown under high and low light. *British Phycological Journal*. 17(2): 169-182.

Foyer, H. C.; Bloom, J. A.; Queval, G. and Noctor, G. (2009). Photorespiratory metabolism: genes, mutants, energetics, and redox signaling. *Annual Review of Plant Biology*. 60: 455-484.

Francisco, A. J.; Cerveny, G. C.; Meyer, L. D.; Mixan, J. B.; Klussman, K.; Chace, D. F.; Rejniak, S. X.; Gordon, K. A.; DeBlanc, R.; Toki, B. E.; Law, C.-L.; Doronina, S. O.; Siegall, C. B.; Senter, P. D. and Wahl, A. F. (2003). cAC10-vcMMAE, an anti-CD30–monomethyl auristatin E conjugate with potent and selective antitumor activity. *Blood*. 102(4): 1458-1465.

Frankmölle, P. W.; Knuebel, G.; Moore, E. R. and Patterson, M. L. G. (1992a). Antifungal cyclic peptides from the terrestrial blue-green alga *Anabaena laxa*. II. Structures of Laxaphycins A, B, D and E. *The Journal of Antibiotics*. 45(9): 1458-1466.

Frankmölle, P. W.; Larsen, K. L.; Caplan, R. F.; Patterson, M. L. G.; Knübel, G.; Levine, A. I. and Moore, E. R. (1992b). Antifungal cyclic peptides from the terrestrial blue-green alga *Anabaena* laxa. I. Isolation and biological properties. *The Journal of Antibiotics*. 45(9): 1451-1457.

Fricke, N. (2006). Untersuchungen zur molekulargenetischen aund chemotaxonomischer vietnamsischer cyanobacterirenstämme. Greifswald, *EMAU Greifswald*. Diploma thesis.

Friedmann, E. I. and Galun, M. (1974). Desert algae, lichens and fungi. Desert Biology. Brown G. W. J. New York: *Academic Press*. 165-212.

Fujii, K.; Sivonen, K.; Adachi, K.; Noguchi, K.; Shimizu, Y.; Sano, H.; Hirayama, K.; Suzuki, M. and Harada, K.-I. (1997). Comparative study of toxic and non-toxic cyanobacterial products: A novel glycoside, suomilide, from non-toxic *Nodularia spumigena* HKVV. *Tetrahedron Letters*. 38(31): 5529-5532.

Furey, A.; Crowley, J.; Shuilleabhain, A. N.; Skulberg, O. M. and James, K. J. (2003). The first identification of the rare cyanobacterial toxin, homoanatoxin-a, in Ireland. *Toxicon*. 41(3): 297-303.

Gademann, K. and Portmann, C. (2008). Secondary metabolites from cyanobacteria: complex structures and powerful bioactivities. *Current Organic Chemistry*. 12(4).

Galhano, V.; de Figueiredo, R. D.; Alves, A.; Correia, A.; Pereira, M. J.; Gomes-Laranjo, J. and Peixoto, F. (2011). Morphological, biochemical and molecular characterization of *Anabaena*, *Aphanizomenon* and *Nostoc* strains (Cyanobacteria, Nostocales) isolated from Portuguese freshwater habitats. *Hydrobiologia*. 663: 187-203.

Gallis, A. H.; Drew, H. R. and Pickard, W. W. (1990). Amphotericin B: 30 years of clinical experience. *Review of Infectious Diseases*. 12(2): 308-329.

Gantar, M.; Berry, J. P.; Thomas, S.; Wang, M.; Perez, R.; Rein, K. S. and King, G. (2008). Allelopathic activity among cyanobacteria and microalgae isolated from Florida freshwater habitats. *FEMS Microbiology Ecology*. 64(1): 55-64.

Gbankoto, A.; Vigo, J.; Dramane, K.; Banaigs, B.; Aina, E. and Salmon, J. M. (2005). Cytotoxic effect of Laxaphycins A and B on human lymphoblastic cells (CCRF-CEM) using digitised videomicrofluorometry. *In Vivo*. 19(3): 577-582.

Gearhart, M. O. (1994). Worsening of liver function with fluconazole and review of azole antifungal hepatotoxicity. *The Annals of Pharmacotherapy*. 28(10): 1177-1181.

Geitler, L. (1932). In Synoptische Darstellung der Cyanophyceen in morphologischer und systematischer Hinsicht. *Beihefte zum Botanischen Centralblatt*. 163-324.

Gerwick, H. W.; Jiang, D. Z.; Agarwal, K. S. and Farmer, T. B. (1992). Total structure of hormothamnin A, a toxic cyclic undecapeptide from the tropical marine cyanobacterium *Hormothamnion enteromorphoides*. *Tetrahedron*. 48(12): 2313-2324.

Gerwick, H. W. and Moore, S. B. (2012). Lessons from the past and charting the future of marine natural products drug discovery and chemical biology. *Chemistry & Biology*. 19(1): 85-98.

Gerwick, W.; Roberts, M.; Proteau, P. and Chen, J. L. (1994). Screening cultured marine microalgae for anticancer-type activity. *Journal of Applied Phycology*. 6(2): 143-149.

Gerwick, W. H.; Mrozek, C.; Moghaddam, M. F. and Agarwal, S. K. (1989). Novel cytotoxic peptides from the tropical marine cyanobacterium *Hormothamnion enteromorphoides* 1. Discovery, isolation and initial chemical and biological characterization of the hormothamnins from wild and cultured material. *Cellular and Molecular Life Sciences*. 45(2): 115-121.

Gerwig, J. G.; Kamerling, P. J. and Vliegenthart, F. G. J. (1978). Determination of the d and l configuration of neutral monosaccharides by high-resolution capillary G.L.C. *Carbohydrate Research*. 62(2): 349-357.

Ghasemi, Y.; Yazdi, M. T.; Shokravi, S.; Soltani, N. and Zarrini, G. (2003). Antifungal and antibacterial activity of paddy-fields cyanobacteria from the North of Iran. *Journal of Sciences, Islamic Republic of Iran*. 14(3): 203-209.

Ghazala, B. and Shameel, M. (2005). Phytochemistry and bioactivity of some freshwater green algae from Pakistan. *Pharmaceutical Biology*. 43(4): 358-369.

Gibson, C. E. and Foy, R. H. (1983). The photosynthesis and growth efficiency of a planktonic blue-green alga, *Oscillatoria redekei*. *British Phycological Journal*. 18(1): 39-45.

Giovannoni, S. J.; Turner, S.; Olsen, G. J.; Barns, S.; Lane, D. J. and Pace, N. R. (1988). Evolutionary relationships among cyanobacteria and green chloroplasts. *Journal of Bacteriology*. 170(8): 3584-3592.

Girmenia, C. and Finolezzi, E. (2011). New-generation triazole antifungal drugs: review of the Phase II and III trials. *Clinical Investigation*. 1(11): 1577-1594.

Gleason, F. K. and Wood, J. M. (1976). Ribonucleotide reductase in blue-green algae: Dependence on adenosylcobalamin. *Science*. 192: 1343-1344.

Gleason, F. K. and Frick, T. D. (1980). Adenosylcobalamin-dependent ribonucleotide reductase from the blue-green alga, *Anabaena* sp. *The Journal of Biological Chemistry*. 255(16): 7728-7733.

Gleason, K. F. and Olszewski, E. N. (2002). Isolation of the gene for the B_{12}-dependent ribonucleotide reductase from *Anabaena* sp. strain PCC 7120 and expression in *Escherichia coli*. *Journal of Bacteriology*. 184(23): 6544–6550.

Grindberg, V. R.; Shuman, F. C.; Sorrels, M. C.; Wingerd, J. and Gerwick, H. W. (2008). Neurotoxic alkaloids from cyanobacteria. Morden alkaloids: structure, isolation, synthesis and biology. Fattorusso E. and Taglialatela-Scafati O.: *Wiley-VCH*.

Groll, H. A.; Piscitelli, C. S. and Walsh, J. T. (1998). Clinical pharmacology of systemic antifungal agents: A comprehensive review of agents in clinical use, current investigational compounds, and putative targets for antifungal drug development. *Advances in Pharmacology*. 44: 343-500.

Groll, H. A. and Walsh, J. T. (2001). Caspofungin: pharmacology, safety and therapeutic potential in superficial and invasive fungal infections. *Expert Opinion on Investigational Drugs*. 10(8): 1545-1558.

Gupta, V.; Prasanna, R.; Natarajan, C.; Srivastava, A. K. and Sharma, J. (2010). Identification, characterization, and regulation of a novel antifungal chitosanase gene (cho) in *Anabaena* spp. *Applied and Environmental Microbiology*. 76(9): 2769-2777.

Gupta, V.; Natarajan, C.; Kumar, K. and Prasanna, R. (2011). Identification and characterization of endoglucanases for fungicidal activity in *Anabaena laxa* (Cyanobacteria). *Journal of Applied Phycology*. 23(1): 73-81.

Gupta, V.; Natarajan, C.; Chaudhary, V.; Kumar, A.; Sharma, E.; Sharma, J.; Bhatnagar, A. and Prasanna, R. (2012). Analyses of diversity among fungicidal *Anabaena* strains. *Journal of Applied Phycology*. 24: 1395–1405.

Guyot, M.; Doré, J. C. and Devillers, J. (2004). Typology of secondary cyanobacterial metabolites from minimum spanning tree analysis. *SAR and QSAR in Environmental Research*. 15(2): 101-114.

Hajdu, S.; Höglander, H. and Larsson, U. (2007). Phytoplankton vertical distributions and composition in Baltic Sea cyanobacterial blooms. *Harmful Algae*. 6(2): 189-205.

Halinen, K.; Jokela, J.; Fewer, P. D.; Wahlsten, M. and Sivonen, K. (2007). Direct evidence for production of microcystins by *Anabaena* strains from the Baltic Sea. *Applied and Environmental Microbiology*. 73(20): 6543–6550.

Halinen, K.; Fewer, D. P.; Sihvonen, L. M.; Lyra, C.; Eronen, E. and Sivonen, K. (2008). Genetic diversity in strains of the genus *Anabaena* isolated from planktonic and benthic habitats of the Gulf of Finland (Baltic Sea). *FEMS Microbiology Ecology*. 64(2): 199-208.

Hällfors, G. (2004). Checklist of Baltic Sea phytoplankton species. Baltic Sea Environment Proceedings. HETCOM - Helsinki Commission Baltic Marine Environment Protection Commission. 95.

Harada, K.-I.; Kimura, Y.; Ogawa, K.; Suzuki, M.; Dahlem, M. A.; Beasley, R. V. and Carmichael, W. W. (1989). A new procedure for the analysis and purification of naturally occurring anatoxin-a from the blue-green alga *Anabaena flos-aquae*. *Toxicon*. 27(12): 1289-1296.

Harada, K.-I.; Ogawa, K.; Kimura, Y.; Murata, H.; Suzuki, M.; Thorn, M. P.; Evans, R. W. and Carmichael, W. W. (1991). Microcystins from *Anabaena flos-aquae* NRC 525-17. *Chemical Research in Toxicology*. 4(5): 535-540.

Harada, K.-I.; Fujii, K.; Shimada, T.; Suzuki, M.; Sano, H.; Adachi, K. and Carmichael, W. W. (1995). Two cyclic peptides, anabaenopeptins, a third group of bioactive compounds from the cyanobacterium *Anabaena flos-aquae* NRC 525-17. *Tetrahedron Letters*. 36(9): 1511-1514.

Harada, K.-I. (2004). Production of secondary metabolites by freshwater cyanobacteria. *Chemical & Pharmaceutical Bulletin*. 82(8): 889-899.

Harvey, A. (2008). Natural product pharmaceuticals: A diverse approach to drug discovery. *Drug Discovery Today*. 13(19/20): 894-901.

Hashimoto, E.; Yabuta, Y.; Takenaka, S.; Yamaguchi, Y.; Takenaka, H. and Watanabe, F. (2012). Characterization of corrinoid compounds from edible cyanobacterium *Nostochopsis* sp. *Journal of Nutritional Science and Vitaminology* 58: 50-53.

Hashtroudi, M. S.; Ghassempour, A.; Riahi, H.; Shariatmadari, Z. and Khanjir, M. (2013). Endogenous auxins in plant growth-promoting Cyanobacteria—*Anabaena vaginicola* and *Nostoc calcicola*. *Journal of Applied Phycology*. 25(2): 379-386.

Heinek, S. (2011). Phytochemische und biologische Untersuchung des vietnamesischen Cyanobakteriums *Anabaena* sp. Greifswald, *EMAU Greifswald*. Master of Science in Engineering.

Henson, J. B.; Watson, E. L. and Barnum, R. S. (2002). Molecular differentiation of the heterocystous cyanobacteria, *Nostoc* and *Anabaena*, based on complete *nif*D sequences. *Current Microbiology*. 45(3): 161-164.

Herfindal, L.; Oftedal, L.; Selheim, F.; Wahlsten, M.; Sivonen, K. and Døskeland, S. O. (2005). A high proportion of Baltic Sea benthic cyanobacterial isolates contain apoptogens able to induce rapid death of isolated rat hepatocytes. *Toxicon*. 46(3): 252-260.

Hobson, P. and Fallowfield, H. J. (2003). Effect of irradiance, temperature and salinity on growth and toxin production by *Nodularia spumigena*. *Hydrobiologia*. 493(1): 7-15.

Howarth, R. W.; Marino, R. and Cole, J. J. (1988). Nitrogen fixation in freshwater, estuarine and marine ecosystems. 2. Biogeochemical controls. *Limnology and Oceanography*. 33: 688-701.

Hrouzek, P. (2010). Cytotoxicity and secondary metabolites production in cyanobacteria. České Budějovice, *University of South Bohemia*. Dissertation.

Huber, L. A. (1985). Factors affecting the germination of akinetes of *Nodularia spumigena* (Cyanobacteriaceae). *Applied and Environmental Microbiology*. 49(1): 73-78.

Huebel, H. (1984). Die Bindung des Molekularen Stickstoffs durch Autotrophe und Heterotrophe Mikroorganismen in Kustengewassern der DDR und an Einigen Stationen der Ostsee. *Ernst-Moritz-Arndt-Univ. Greifswald*. Dissertation.

ICH (1996). Validation of analytical procedures: Text and methodology Q2(R1). International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. Geneva.

Imamura, N. (1981). Studies on the water blooms in Lake Kasumigaura. *Verhandlungen der internationalen Vereinigung für theoretische und angewandte Limnologie*. 21: 652-658.

Ishibashi, M. (1986). Scytophycins, cytotoxic and antimycotic agents from the cytcinophyte *Scytonema pseudshofmanni*. *The Journal of Organic Chemistry*. 51: 5300-5306.

Ishida, K.; Murakami, M.; Matsuda, H. and Yamaguchi, K. (1995). Micropeptin 90, a plasmin and trypsin inhibitor from the blue-green alga *Microcystis aeruginosa* (NIES-90). *Tetrahedron Letters*. 36(20): 3535-3538.

Ishida, K.; Matsuda, H. and Murakami, M. (1998). Four new microginins, linear peptides from the cyanobacterium Microcystis aeruginosa. *Tetrahedron*. 54(44): 13475-13484.

Iwatani, W.; Arika, T. and Yamaguchi, H. (1993). Two mechanisms of butenafine action in *Candida albicans*. *Antimicrobial Agents and Chemotherapy*. 37(4): 785–788.

Jaki, B.; Orjala, J. and Sticher, O. (1999). A novel extracellular diterpenoid with antibacterial activity from the cyanobacterium *Nostoc commune*. *Journal of Natural Products*. 62(3): 502-503.

Jaki, B.; Orjala, J.; Heilmann, J.; Linden, A.; Vogler, B. and Sticher, O. (2000). Novel extracellular diterpenoids with biological activity from the cyanobacterium *Nostoc commune*. *Journal of Natural Products*. 63(3): 339-343.

Jaki, B.; Zerbe, O.; Heilmann, J. and Sticher, O. (2001). Two novel cyclic peptides with antifungal activity from the cyanobacterium *Tolypothrix byssoidea* (EAWAG 195). *Journal of Natural Products*. 64(2): 154-158.

Janson, S. and Granéli, E. (2002). Phylogenetic analyses of nitrogen-fixing cyanobacteria from the Baltic Sea reveal sequence anomalies in the phycocyanin operon. *International Journal of Systematic and Evolutionary Microbiology*. 52 1397–1404.

Jarvis, B.; Figgitt, P. D. and Scott, J. L. (2004). Micafungin. *Drugs*. 64(9): 969-982.

Jones, J. and Sivonen, K. (1999). Cyanobacterial toxins. Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management. Chorus I. and Bartram J. London: *E & FN SPON*. 41–111.

Jung, H. J.; Moore, E. R. and Patterson, M. L. G. (1991). Scytophycins from a blue-green alga belonging to the Nostocaceae. *Phytochemistry*. 30(11): 3615-3616.

Kaebernick, M.; Neilan, A. B.; Boerner, T. and Dittmann, E. (2000). Light and the transcriptional response of the microcystin biosynthesis gene cluster. *Applied and Environmental Microbiology*. 66(8): 3387-3392.

Kajiyama, S. I.; Kanzaki, H.; Kawazu, K. and Kobayashi, A. (1998). Nostofungicidine, an antifungal lipopeptide from the field-grown terrestrial blue-green alga *Nostoc commune*. *Tetrahedron Letters*. 39(22): 3737-3740.

Karlsson, M. K.; Kankaanpää, H.; Huttunen, M. and Meriluoto, J. (2005). First observation of microcystin-LR in pelagic cyanobacterial blooms in the northern Baltic Sea. *Harmful Algae*. 4(1): 163-166.

Kashyap, S.; Garg, V.; Sharma, P.; Kumar, N.; Dudhe, R. and Gupta, J. (2012). Thiazoles: Having diverse biological activities. *Medicinal Chemistry Research*. 21(8): 2123-2132.

Kehr, J. C.; Picchi, D. G. and Dittmann, E. (2011). Natural product biosyntheses in cyanobacteria: A treasure trove of unique enzymes. *Beilstein Journal of Organic Chemistry*. 7: 1622-1635.

Kim, H.; Lantvit, D.; Hwang, C. H.; Kroll, D. J.; Swanson, S. M.; Franzblau, S. G. and Orjala, J. (2012). Indole alkaloids from two cultured cyanobacteria, *Westiellopsis* sp. and *Fischerella muscicola*. *Bioorganic & Medicinal Chemistry*. 20(17): 5290-5295.

Kim, J.-D. (2006). Screening of cyanobacteria (blue-green algae) from rice paddy soil for antifungal activity against plant pathogenic fungi. *Mycrobiology*. 34(3): 138-142.

Kingston, D. G. I.; Rao, M. M.; Spittler, T. D.; Pettersen, R. C. and Cullen, D. L. (1975). Sesquiterpenes from *Flourensia cernua*. *Phytochemistry.* . 14: 2033-2037.

Klochenko, P. D.; Elanskaya, I. A.; Shevchenko, T. F. and Sokolova, E. V. (2001). Antifungal activity of freshwater cyanobacteria. *Archiv für Hydrobiologie*. 140: 143-149.

Kobayashi, M. and Shimizu, S. (1999). Cobalt proteins. *European Journal of Biochemistry*. 261(1): 1-9.

Kodani, S.; Ishida, K. and Murakami, M. (1998). Dehydroradiosumin, a trypsin inhibitor from the cyanobacterium *Anabaena cylindrica*. *Journal of Natural Products*. 61(6): 854-856.

Komárek, J. and Anagnostidis, K. (1989). Modern approach to the classification system of Cyanophytes 4 - Nostocales. *Algological Studies/Archiv für Hydrobiologie, Supplement Volumes*. 56: 247-345.

Komárek, J. (2005). The modern classification of cyanoprokaryotes (cyanobacteria). *Oceanological and Hydrobiological Studies* XXXIV(Supplement 3).

(2006). Cyanobacterial taxonomy: Current problems and prospects for the integration of traditional and molecular approaches. *Algae*. 21(4): 349-375.

(2010). Recent changes (2008) in cyanobacteria taxonomy based on a combination of molecular background with phenotype and ecological consequences (genus and species concept). *Hydrobiologia*. 639(1): 245-259.

(2011). Some current problems of modern cyanobacterial taxonomy. Introduction to the 18th IAC Symposium in C˘eske´ Bude˘jovice 2010. Czech Republic, *Fottea*. 11: 1-7.

Komárek, J. and Mareš, J. (2012). An update to modern taxonomy (2011) of freshwater planktic heterocytous cyanobacteria. *Hydrobiologia*. 698(1): 327-351.

Konopka, A. and Brock, T. D. (1978). Effect of temperature on blue-green algae (cyanobacteria) in Lake Mendota. *Appiled and Environmental Microbiology*. 36(4): 572-576.

Kontoyiannis, D. P. and Lewis, R. E. (2002). Antifungal drug resistance of pathogenic fungi. *The Lancet*. 359(9312): 1135-1144.

Kreitlow, S.; Mundt, S. and Lindequist, U. (1999). Cyanobacteria—a potential source of new biologically active substances. *Journal of Biotechnology* 70 61–63.

Krüger, G. H. J. and Eloff, J. N. (1978). The effect of temperature on specific growth rate and activation energy of *Microcystis* and *Synechococcus* isolates relevant to the onset of natural blooms. *Journal of the Limnological Society of Southern Africa*. 4(1): 9-20.

Kurmayer, R. (2011). The toxic cyanobacterium *Nostoc* sp. strain 152 produces highest amounts of microcystin and nostophycin under stress conditions. *Journal of Phycology*. 47(1): 200-207.

Kuse, E. R.; Chetchotisakd, P.; da Cunha, C. A.; Ruhnke, M.; Barrios, C.; Raghunadharao, D.; Sekhon, J. S.; Freire, A.; Ramasubramanian, V.; Demeyer, I.; Nucci, M.; Leelarasamee,

A.; Jacobs, F.; Decruyenaere, J.; Pittet, D.; Ullmann, J. A.; Ostrosky-Zeichner, L.; Lortholary, O.; Koblinger, S.; Diekmann-Berndt, H. and Cornely, A. O. (2007). Micafungin versus liposomal amphotericin B for candidaemia and invasive candidosis: a phase III randomised double-blind trial. *The Lancet*. 369(9572): 1519-1527.

Kuti, E. L. and Kuti, J. L. (2010). Pharmacokinetics, antifungal activity and clinical efficacy of anidulafungin in the treatment of fungal infections. *Expert Opinion on Drug Metabolism & Toxicology*. 6(10): 1287-1300.

Laamanen, J. M.; Gugger, M. F.; Lehtimäki, J. M.; Haukka, K. and Sivonen, K. (2001). Diversity of toxic and nontoxic *Nodularia* isolates (cyanobacteria) and filaments from the Baltic Sea. *Applied and Environmental Microbiology*. 67(10): 4638-4647.

Laamanen, M. J.; Forsström, L. and Sivonen, K. (2002). Diversity of *Aphanizomenon flosaquae* (cyanobacterium) populations along a Baltic Sea salinity gradient. *Applied Environmental Microbiology*. 68(11): 5296-5303.

Lachance, M.-A. (1981). Genetic relatedness of heterocystous cyanobacteria by deoxyribonucleic acid-deoxyribonucleic acid reassociation. *International journal of systematic bacteriology*. 31(2): 139-147.

Larsen, L. K.; Moore, R. E. and Patterson, G. M. (1994). beta-Carbolines from the blue-green alga *Dichothrix baueriana*. *Journal of Natural Products*. 57(3): 419-421.

Laurienzo, P. (2010). Marine polysaccharides in pharmaceutical applications: An overview. *Marine Drugs*. 8(9): 2435-2465.

Lawton, L. A.; Edwards, C. and Codd, G. A. (1994). Extraction and high-performance liquid chromatographic method for the determination of microcystins in raw and treated waters. *Analyst*. 119.

Le, T. A. T. (2010). Chemical and biological investigations of Vietnamese cyanobacteria. Pharmaceutical Biology, *University Greifswald*. Dissertation.

Leão, P. N.; Vasconcelos, M. T. S. D. and Vasconcelos, V. M. (2007). Role of marine cyanobacteria in trace metal bioavailability in seawater. *Microbial Ecology*. 53(1): 104-109.

Leão, P. N.; Vasconcelos, M. T. S. D. and Vasconcelos, V. M. (2009). Allelopathy in freshwater cyanobacteria. *Critical Reviews in Microbiology*. 35(4): 271-282.

Lehtimaeki, J.; Lyra, C.; Suomalainen, S.; Sundman, P.; Rouhiainen, L.; Lars Paulin; Salkinoja-Salonen, M. and Sivonen, K. (2000). Characterization of *Nodularia* strains, cyanobacteria from brackish waters, by genotypic and phenotypic methods. *International Journal of Systematic and Evolutionary Microbiology*. 50: 1043–1053.

Lehtimäki, J.; Moisander, P.; Sivonen, K. and Kononen, K. (1997). Growth, nitrogen fixation, and nodularin production by two Baltic Sea cyanobacteria. *Applied and Environmental Microbiology*. 63(5): 1647-1656.

Lehtimäki, J. (2000). Characterisation of cyanobacterial strains originating from the Baltic Sea with emphasis on *Nodularia* and its toxin, nodularin. *University of Helsinki, Finland*. Academic dissertation.

Li, R. K. and Rinaldi, M. G. (1999). *In vitro* antifungal activity of nikkomycin Z in combination with fluconazole or itraconazole. *Antimicrobial Agents Chemotherapy*. 43(6): 1401-1405.

Liang, H. (2008). Sordarin, an antifungal agent with a unique mode of action. *Beilstein Journal of Organic Chemistry*. 4(31).

Liere, V. L. and Walsby, A. E. (1982). Chapter 2. Interations of cyanobacteria with light. The biology of cyanobacteria. Carr N. G. and Whitton B. A. Berkeley & Los Angeles: *University of California Press*. 19: 688.

Lightbody, J. J. and Krogmann, W. D. (1967). Isolation and properties of plastocyanin from *Anabaena variabilis*. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*. 131(3): 508-515.

Lincoln, R. A.; Strupinski, K. and Walker, J. M. (1996). The use of *Artemia nauplii* (brine shrimp larvae) to detect toxic compounds from microalgal cultures. *Pharmaceutical Biology*. 34(5): 384-389.

Liu, L. and Rein, S. K. (2010). New peptides isolated from *Lyngbya* species: A review. *Marine Drugs*. 8(6): 1817-1837.

Lopes, V. R. and Vasconcelos, V. M. (2011). Bioactivity of benthic and picoplanktonic estuarine cyanobacteria on growth of photoautotrophs: Inhibition versus stimulation. *Marine Drugs*. 9(5): 790-802.

Ludwig, W.; Strunk, O.; Westram, R.; Richter, L.; Meier, H.; Yadhukumar; Buchner, A.; Tina Lai; Steppi, S.; Jobb, G.; Förster, W.; Brettske, I.; Gerber, S.; Ginhart, A. W.; Gross, O.; Grumann, S.; Hermann, S.; Jost, R.; König, A.; Liss, T.; Lüßmann, R.; May, M.; Nonhoff, B.; Reichel, B.; Strehlow, R.; Stamatakis, A.; Stuckmann, N.; Vilbig, A.; Lenke, M.; Ludwig, T.; Bode, A. and Schleifer, K.-H. (2004). ARB: a software environment for sequence data. *Nucleic Acids Research* 32(4): 1363–1371.

Luesch, H.; Moore, R. E.; Paul, V. J.; Mooberry, S. L. and Corbett, T. H. (2001). Isolation of Dolastatin 10 from the marine cyanobacterium *Symploca* species VP642 and total stereochemistry and biological evaluation of its analogue Symplostatin 1. *Journal of Natural Products*. 64(7): 907-910.

MacMillan, J. B.; Ernst-Russell, M. A.; de Ropp, J. S. and Molinski, T. F. (2002). Lobocyclamides A−C, lipopeptides from a cryptic cyanobacterial mat containing *Lyngbya confervoides*. *The Journal of Organic Chemistry*. 67(23): 8210-8215.

Mahmood, N. A.; Carmichael, W. W. and Pfahler, D. (1988). Anticholinesterase poisonings in dogs from a cyanobacterial (blue-green algae) bloom dominated by *Anabaena flos-aquae*. *Am. J. Vet. Res.* 49(4): 500-503.

Mankiewicz, J.; Tarczyn'ska, M.; Walter, Z. and Zalewski, M. (2003). Natural toxins from cyanobacteria. *ACTA Biologica cracoviensia Series Botanica* 45(2): 9-20.

Marinelli, F. (2009). Microbial products to novel drugs that target a multitude of disease indications. Methods in Enzymology. David A. H., *Academic Press*. Volume 458: 29-58.

Marquez, B. L.; Watts, K. S.; Yokochi, A.; Roberts, M. A.; Verdier-Pinard, P.; Jimenez, J. I.; Hamel, E.; Scheuer, P. J. and Gerwick, W. H. (2002). Structure and absolute stereochemistry of hectochlorin, a potent stimulator of actin assembly. *Journal of Natural Products*. 65(6): 866-871.

Martens, J. H.; Barg, H.; Warren, M. and Jahn, D. (2002). Microbial production of vitamin B12. *Applied Microbiology and Biotechnology*. 58(3): 275-285.

Martínez-Luis, S.; Pérez-Vásquez, A. and Mata, R. (2007). Natural products with calmodulin inhibitor properties. *Phytochemistry*. 68(14): 1882-1903.

Martins, R. F.; Ramos, M. F.; Herfindal, L.; Sousa, J. A.; Skærven, K. and Vasconcelos, V. M. (2008). Antimicrobial and cytotoxic assessment of marine cyanobacteria - *Synechocystis and Synechococcus*. *Marine Drugs*. 6(1): 1–11.

Mata, R.; Bye, R.; Linares, E.; Martha, M.; Rivero-Cruz, I.; Pérez, O. and Timmermann, B. N. (2003). Phytotoxic compounds from *Flourensia cernua*. *Phytochemistry*. 64(1): 285-291.

Matsunaga, S.; Moore, R. E.; Niemczura, W. P. and Carmichael, W. W. (1989). Anatoxin-a(s), a potent anticholinesterase from *Anabaena flos-aquae*. *Journal of the American Chemical Society*. 111(20): 8021-8023.

Matsunaga, T.; Takeyama, H.; Miyashita, H. and Yokouchi, H. (2005). Marine Microalgae. Marine Biotechnology I. Ulber R. and Gal Y., *Springer Berlin Heidelberg*. 96: 165-188.

Matthew, S.; Paul, V. J. and Luesch, H. (2009). Largamides A − C, tiglic acid-containing cyclodepsipeptides with elastase-inhibitory activity from the marine cyanobacterium *Lyngbya confervoides*. *Planta Medica*. 75(5): 528–533.

Mazur-**Marzec, H.; Żeglińska, L. and Pliński, M. (2005).** The effect of salinity on the growth, toxin production, and morphology of *Nodularia spumigena* isolated from the Gulf of Gdańsk, southern Baltic Sea. *Journal of Applied Phycology*. 17(2): 171-179.

McCalley, D. V. (2005). Choice of buffer for the analysis of basic peptides in reversed-phase HPLC. *LCGC Asia Pacific*. 8(1).

Medina-Jaritz, N. B.; Perez-Solis, D. R.; Ruiloba de Leon, S. L. F. and Olvera-Ramírez, R. (2011). Antimicrobial activity of aqueous and methanolic extracts from *Arthrospira maxima*. Science against microbial pathogens: communicating current research and technological advances. Méndez-Vilas A. 1267-1271.

Menichetti, F. (2009). Anidulafungin, a new echinocandin: Effectiveness and tolerability. *Drugs*. 69: 95-97.

Milligan, K. E.; Marquez, B. L.; Williamson, T. R. and Gerwick, W. H. (2000). Lyngbyabellin B, a toxic and antifungal secondary metabolite from the marine cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products*. 63(10): 1440-1443.

Mo, S.; Krunic, A.; Chlipala, G. and Orjala, J. (2009). Antimicrobial ambiguine isonitriles from the cyanobacterium *Fischerella ambigua*. *Journal of Natural Products*. 72(5): 894-899.

Moffitt, M. C. and Neilan, B. A. (2001). On the presence of peptide synthetase and polyketide synthase genes in the cyanobacterial genus *Nodularia*. *FEMS Microbiology Letters*. 196(2): 207-214.

Moffitt, M. C. and Neilan, B. A. (2004). Characterization of the nodularin synthetase gene cluster and proposed theory of the evolution of cyanobacterial hepatotoxins. *Appiled and Environmental Microbiology*. 70(11): 6353-6362.

Mohlin, M. (2010). On the ecophysiology of Baltic cyanobacteria, focusing on bottom-up factors. *University of Gothenburg*. Doctoral Thesis.

Monde, K.; Sasaki, K.; Shirata, A. and Takasugi, M. (1991). Brassicanal C and two dioxindoles from cabbage. *Phytochemistry*. 30: 2915-2917.

Moon, S. S.; Chen, J. L.; Moore, R. E. and Patterson, G. M. L. (1992). Calophycin, a fungicidal cyclic decapeptide from the terrestrial blue-green alga *Calothrix fusca*. *The Journal of Organic Chemistry*. 57(4): 1097-1103.

Moore, B. S.; Chen, J. L.; Patterson, G. M. L.; Moore, R. E.; Brinen, L. S.; Kato, Y. and Clardy, J. (1990). [7.7]Paracyclophanes from blue-green algae. *Journal of the American Chemical Society*. 112(10): 4061-4063.

Moore, E. R.; Cheuk, C.; Yang, G. X. Q.; Patterson, M. L. G.; Bonjouklian, R.; Smitka, T. A.; Mynderse, J. S.; Foster, S. R. and Jones, D. N. (1987a). Hapalindoles, antibacterial and antimycotic alkaloids from the cyanophyte *Hapalosiphon fontinalis*. *The Journal of Organic Chemistry*. 52(6): 1036-1043.

Moore, E. R.; Yang, G. X. Q. and Patterson, M. L. G. (1987b). Fontonamide and anhydrohapaloxindole A, two new alkaloids from the blue-green alga *Hapalosiphon fontinalis*. *The Journal of Organic Chemistry*. 52(17): 3773-3777.

Moore, R. E. (1982). Toxins, anticancer agents, and tumor promoters from marine prokaryotes. *Pure and Applied Chemistry*. 54(10): 1919-1934.

Moore, R. E.; Patterson, G. M. L.; Mynderse, J. S.; Barchi, J.; Norton, T. R.; Furusawa, E. and Furusawa, S. (1986). Toxins from cyanophytes belonging to the Scytonemataceae. *Pure and Applied Chemistry*. 58(2): 263-271.

Moore, R. E.; Cheuk, C.; Yang, X. Q. G.; Patterson, G. M. L.; Bonjouklian, R.; Smitka, T. A.; Mynderse, J. S.; Foster, R. S. and Jones, N. D. (1987c). Hapalindoles, antibacterial and antimycotic alkaloids from the cyanophyte *Hapalosiphon fontinalis*. *The Journal of Organic Chemistry*. 52(6): 1036-1043.

Moore, R. E. (1996). Cyclic peptides and depsipeptides from cyanobacteria: A review. *Journal of Industrial Microbiology & Biotechnology*. 16(2): 134-143.

Moreira, I. C.; Lago, J. H. G.; Young, M. C. M. and Roque, N. F. (2003). Antifungal aromadendrane sesquiterpenoids from the leaves of *Xylopia brasiliensis*. *Journal of the Brazilian Chemical Society*. 14: 828-831.

Mundt, S.; Kreitlow, S.; Nowotny, A. and Effmert, U. (2001). Biochemical and pharmacological investigations of selected cyanobacteria. *International Journal of Hygiene and Environmental Health*. 203(4): 327-334.

Mundt, S.; Kreitlow, S.; Jansen, R. and Mundt, S. (2003). Fatty acids with antibacterial activity from the cyanobacterium *Oscillatoria redekei* HUB 051. *Journal of Applied Phycology*. 15 263–267.

Muyzer, G.; De Waal, E. C. and Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*. 59(3): 695–700.

Muyzer, G.; Teske, A.; Wirsen, C. O. and Jannasch, H. W. (1995). Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Archives of Microbiology*. 164(3): 165-172.

Namikoshi, M. and Rinehart, K. L. (1996). Bioactive compounds produced by cyanobacteria. *Journal of Industrial Microbiology & Biotechnology*. 17(5): 373-384.

Nayak, S.; Prasanna, R.; Prasanna, B. and Sahoo, D. (2007). Analysing diversity among Indian isolates of *Anabaena* (Nostocales, Cyanophyta) using morphological, physiological and biochemical characters. *World Journal of Microbiology and Biotechnology*. 23(11): 1575-1584.

Negri, A. P. and Jones, G. J. (1995). Bioaccumulation of paralytic shellfish poisoning (PSP) toxins from the cyanobacterium *Anabaena circinalis* by the freshwater mussel *Alathyria condola*. *Toxicon*. 33(5): 667-678.

Neilan, B. A.; Jacobs, D. and Goodman, A. E. (1995). Genetic diversity and phylogeny of toxic cyanobacteria determined by DNA polymorphisms within the phycocyanin locus. *Applied and Environmental Microbiology*. 61(11): 3875-3883.

Neilan, B. A.; Pearson, L. A.; Moffitt, M. C.; Mihali, K. T.; Kaebernick, M.; Kellmann, R. and Pomati, F. (2008). The genetics and genomics of cyanobacterial toxicity. *Advances in Experimental Medicine and Biology*. 619: 417-452.

Nelissen, B.; Baere, R.; Wilmotte, A. and Wachter, R. (1996). Phylogenetic relationships of nonaxenic filamentous cyanobacterial strains based on 16S rRNA sequence analysis. *Journal of Molecular Evolution*. 42(2): 194-200.

Neuhof, T.; Schmieder, P.; Preussel, K.; Dieckmann, R.; Pham, H.; Bartl, F. and von Döhren, H. (2005). Hassallidin A, a glycosylated lipopeptide with antifungal activity from the cyanobacterium *Hassallia* sp. *Journal of Natural Products*. 68(5): 695-700.

Neuhof, T.; Schmieder, P.; Seibold, M.; Preussel, K. and von Döhren, H. (2006). Hassallidin B—Second antifungal member of the Hassallidin family. *Bioorganic & Medicinal Chemistry Letters*. 16(16): 4220-4222.

Newman, D. J. and Cragg, G. M. (2012). Natural products as sources of new drugs over the 30 years from 1981 to 2010. *Journal of Natural Products*. 75(3): 311-335.

Nicklisch, A. and Kohl, J.-G. (1983). Growth kinetics of *Microcystis aeruginosa* (KÜTZ) KÜTZ as a basis for modelling its population dynamics. *Internationale Revue der gesamten Hydrobiologie und Hydrographie*. 68(3): 317-326.

Niemi, A. (1979). Blue-green algal blooms and N:P ratio in the Baltic Sea *Acta Botanica Fennica*. 110: 57-61.

Nishiwaki-Matsushima, R.; Ohta, T.; Nishiwaki, S.; Suganuma, M.; Kohyama, K.; Ishikawa, T.; Carmichael, W. W. and Fujiki, H. (1992). Liver tumor promotion by the cyanobacterial cyclic

peptide toxin microcystin-LR. *Journal of Cancer Research and Clinical Oncology*. 118(6): 420-424.

Nogle, L. M.; Okino, T. and Gerwick, W. H. (2001). Antillatoxin B, a neurotoxic lipopeptide from the marine cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products*. 64(7): 983-985.

Nordin, R. D. and Stein, J. N. (1980). Taxonomic revision of *Nodularia* (Cyanophyceae / Cyanobacteria). *Canadian Journal of Botany*. 58: 1211-1224.

Nordlund, P. and Reichard, P. (2006). Ribonucleotide reductases. *Annual Review of Biochemistry*. 75(1): 681-706.

Nosanchuk, J. D. (2006). Current status and future of antifungal therapy for systemic mycoses. *Recent Patents on Anti-Infective Drug Discovery*. 1: 75-84.

Novak, J. T. and Brune, D. E. (1985). Inorganic carbon limited growth kinetics of some freshwater algae. *Water Research*. 19(2): 215-225.

Odds, F. C.; Brown, A. J. P. and Gow, N. A. R. (2003). Antifungal agents: Mechanisms of action. *Trends in Microbiology*. 11(6): 272-279.

Onodera, H.; Oshima, Y.; Henriksen, P. and Yasumoto, T. (1997). Confirmation of anatoxin-a(s), in the cyanobacterium *Anabaena lemmermannii*, as the cause of bird kills in Danish Lakes. *Toxicon*. 35(11): 1645-1648.

Ördög, V.; Stirk, W. A.; Lenobel, R.; Bancirova, M.; Strnad, M.; van Staden, J.; Syigeti, J. and Nemeth, L. (2004). Screening microalgae for some potentially useful agricultural and pharmaceutical secondary metabolites. *Journal of Applied Phycology* 16: 309-314.

Orjala, J.; Nagle, D. G.; Hsu, V. and Gerwick, W. H. (1995). Antillatoxin: an exceptionally ichthyotoxic cyclic lipopeptide from the tropical cyanobacterium *Lyngbya majuscula*. *Journal of the American Chemical Society*. 117(31): 8281-8282.

Osswald, J.; Rellán, S.; Gago, A. and Vasconcelos, V. (2007). Toxicology and detection methods of the alkaloid neurotoxin produced by cyanobacteria, anatoxin-a. *Environment International*. 33(8): 1070-1089.

Panosso, R. and Granéli, E. (2000). Effects of dissolved organic matter on the growth of *Nodularia spumigena* (Cyanophyceae) cultivated under N or P deficiency. *Marine Biology*. 136(2): 331-336.

Patterson, G.; Larsen, L. and Moore, R. (1994). Bioactive natural products from blue-green algae. *Journal of Applied Phycology*. 6(2): 151-157.

Patterson, G. M. L.; Baldwin, C. L.; Bolis, C. M.; Caplan, F. R.; Karuso, H.; Larsen, L. K.; Levine, I. A.; Moore, R. E.; Nelson, C. S.; Tschappat, K. D.; Tuang, G. D.; Furusawa, E.; Furusawa, S.; Norton, T. R. and Raybourne, R. B. (1991). Antineoplastic activity of cultured blue-green algae (Cyanophyta). *Journal of Phycology*. 27(4): 530-536.

Pawar, S. T. and Puranik, P. R. (2008). Screening of terrestrial and freshwater halotolerant cyanobacteria for antifungal activities. *World Journal of Microbiology and Biotechnology*. 24(7): 1019-1025.

Pearson, L.; Mihali, T.; Moffitt, M.; Kellmann, R. and Neilan, B. (2010). On the chemistry, toxicology and genetics of the cyanobacterial toxins, microcystin, nodularin, saxitoxin and cylindrospermopsin. *Marine Drugs*. 8(5): 1650-1680.

Pechar, L. (1992). Water blooms of *Aphanizomenon flos-aquae*. An ecological study of fish pond populations. *Archiv für Hydrobiologie - Supplement*. 90: 339-418.

Pedersén, M. and DaSilva, E. J. (1973). Simple brominated phenols in the bluegreen alga *Calothrix brevissima* West. *Planta*. 115(1): 83-86.

Pergament, I. and Carmeli, S. (1994). Schizotrin A: a novel antimicrobial cyclic peptide from a cyanobacterium. *Tetrahedron Letters*. 35(45): 8473-8476.

Pettersen, R. C.; Cullen, D. L.; Spittler, T. D. and Kingston, D. G. I. (1975). The crystal and molecular structure of flourensadiol, a natural product sesquiterpene isolated from a West Texas shrub. *Acta Crystallographica*. B31: 1124.

Pettit, G. R.; Kamano, Y.; Herald, C. L.; Tuinman, A. A.; Boettner, F. E.; Kizu, H.; Schmidt, J. M.; Baczynskyj, L.; Tomer, K. B. and Bontems, R. J. (1987). The isolation and structure of a remarkable marine animal antineoplastic constituent: dolastatin 10. *Journal of the American Chemical Society*. 109(22): 6883-6885.

Pham, H. T. L. (2007). Bioassay-guided isolation of cytotoxic and antifungal compounds from cyanobacteria. *EMAU Greifswald*. Dr. rer. nat.

Piccardi, R.; Frosini, A.; Tredici, M. R. and Margheri, M. C. (2000). Bioactivity in free-living and symbiotic cyanobacteria of the genus *Nostoc*. *Journal of Applied Phycology*. 12(3): 543-547.

Porcs-Makkay, M.; Volk, B.; Kapiller-Dezsófi, R.; Mezei, T. and Simig, G. (2004). New routes to oxindole derivatives. *Monatshefte für Chemie / Chemical Monthly*. 135(6): 697-711.

Post, A. F.; de Wit, R. and Mur, L. R. (1985). Interactions between temperature and light intensity on growth and photosynthesis of the cyanobacterium *Oscillatoria agardhii*. *Journal of Plankton Research*. 7(4): 487-495.

Prasanna, R.; Kumar, R.; Sood, A.; Prasanna, B. M. and Singh, P. K. (2006). Morphological, physiochemical and molecular characterization of *Anabaena* strains. *Microbiological Research*. 161(3): 187-202.

Prasanna, R.; Nain, L.; Tripathi, R.; Gupta, V.; Chaudhary, V.; Middha, S.; Joshi, M.; Ancha, R. and Kaushik, B. D. (2008). Evaluation of fungicidal activity of extracellular filtrates of cyanobacteria – possible role of hydrolytic enzymes. *Journal of Basic Microbiology*. 48(3): 186-194.

Prasanna, R.; Gupta, V.; Natarajan, C. and Chaudhary, V. (2010). Bioprospecting for genes involved in the production of chitosanases and microcystin-like compounds in *Anabaena* strains. *World Journal of Microbiology and Biotechnology*. 26(4): 717-724.

Pruesse, E.; Peplies, J. and Glöckner, F. O. (2012). SINA: accurate high throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics*.

Raab, W. (1978). Mykosebehandlung mit Imidazolderivaten. Berlin-Heidelberg-New York *Springer-Verlag*.

Rai, L. C. and Dubey, S. K. (1989). Impact of chromium and tin on a nitrogen-fixing cyanobacterium *Anabaena doliolum*: Interaction with bivalent cations. *Ecotoxicology and Environmental Safety*. 17(1): 94-104.

Rajaniemi, P.; Hrouzek, P.; Kaštovská, K.; Willame, R.; Rantala, A.; Hoffmann, L.; Komárek, J. and Sivonen, K. (2005). Phylogenetic and morphological evaluation of the genera *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* (Nostocales, Cyanobacteria). *International Journal of Systematic and Evolutionary Microbiology*. 55(1): 11-26.

Ramaswamy, A. V.; Sorrels, C. M. and Gerwick, W. H. (2001). Cloning and biochemical characterization of the hectochlorin biosynthetic gene cluster from the marine cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products*. 70(12): 1977-1986.

Rapala, J.; Sivonen, K.; Luukkainen, R. and Niemelä, S. (1993). Anatoxin-a concentration in *Anabaena* and *Aphanizomenon* under different environmental conditions and comparison of growth by toxic and non-toxic; *Anabaena* -strains — a laboratory study. *Journal of Applied Phycology*. 5(6): 581-591.

Rapala, J.; Sivonen, K.; Lyra, C. and Niemelä, S. I. (1997). Variation of microcystins, cyanobacterial hepatotoxins, in *Anabaena* spp. as a function of growth stimuli. *Applied and Environmental Microbiology*. 63(6): 2206-2212.

Raux, E.; Schubert, H. L. and Warren, M. J. (2000). Biosynthesis of cobalamin (vitamin B_{12}): a bacterial conundrum. *Cellular and Molecular Life Sciences*. 57(13): 1880-1893.

Raveh, A. and Carmeli, S. (2007). Antimicrobial ambiguines from the cyanobacterium *Fischerella* sp. collected in Israel. *Journal of Natural Products*. 70(2): 196-201.

Reichard, P. (1993). From RNA to DNA, why so many ribonucleotide reductases? *Science*. 260: 1773-1777.

Repka, S.; Mehtonen, J.; Vaitomaa, J.; Saari, L. and Sivonen, K. (2001). Effects of nutrients on growth and nodularin production of *Nodularia* strain GR8b. *Microbial Ecology*. 42(4): 606-613.

Rippka, R.; Deruelles, J.; Waterbury, B. J.; Herdman, M. and Stanier, Y. R. (1979). Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *Journal of General Microbiology*. 111(1): 1-61.

Rippka, R.; Castenholz, R. W. and Herdman, M. (2001). Subsection IV. (Formerly Nostocales Castenholz 1989b sensu Rippka, Deruelles, Waterbury, Herdman and Stanier 1979). Bergey's Manual of Systematic Bacteriology. Boone D. R. and Castenholz R. W. New York: *Springer*. 1: 562–566.

Robarts, R. D. and Zohary, T. (1987). Temperature effects on photosynthetic capacity, respiration, and growth rates of bloom-forming cyanobacteria. *New Zealand Journal of Marine and Freshwater Research*. 21(3): 391-399.

Rodriguez-Navarro, A. J.; Lagos, N.; Lagos, M.; Braghetto, I.; Csendes, A.; Hamilton, J.; Figueroa, C.; Truan, D.; Garcia, C.; Rojas, A.; Iglesias, V.; Brunet, L. and Alvarez, F. (2007). Neosaxitoxin as a local anesthetic: Preliminary observations from a first human trial. *Anesthesiology*. 106(2): 339-345.

Rogers, S. D. and Dorsey, J. G. (2000). Chromatographic silanol activity test procedures: the quest for a universal test. *Journal of Chromatography A*. 892(1–2): 57-65.

Rotowa, N. A.; Shadomy, H. J. and Shadomy, S. (1990). In vitro activities of polyene and imidazole antifungal agents against unusual opportunistic fungal pathogens. *Mycoses*. 33(4): 203-211.

Rouhiainen, L.; Paulin, L.; Suomalainen, S.; Hyytiäinen, H.; Buikema, W.; Haselkorn, R. and Sivonen, K. (2000). Genes encoding synthetases of cyclic depsipeptides, anabaenopeptilides, in *Anabaena* strain 90. *Molecular Microbiology*. 37(1): 156-167.

Rounge, T. B.; Rohrlack, T.; Tooming-Klunderud, A.; Kristensen, T. and Jakobsen, K. S. (2007). Comparison of cyanopeptolin genes in *Planktothrix*, *Microcystis,* and *Anabaena* strains: Evidence for independent evolution within each genus. *Applied and Environmental Microbiology*. 73(22): 7322-7330.

Ryder, N. S. (1987). Squalene epoxidase as the target of antifungal allylamines. *Pesticide Science*. 21: 281-288.

Ryley, J. F.; Wilson, R. G.; Gravestock, M. B. and Poyser, J. P. (1981). Experimental approaches to antifungal chemotherapy. *Advances in Pharmacology & Chemotherapy*. 18: 49-176.

Saha, S. K.; Uma, L. and Subramanian, G. (2005). An improved method for marine cyanobacterial DNA isolation. *World Journal of Microbiology and Biotechnology*. 21(6-7): 877-881.

Saito, A. M.; Moffett, W. J.; Chisholm, W. S. and Waterbury, J. B. (2002). Cobalt limitation and uptake in *Prochlorococcus*. *Limnol. Oceanogr.* 47(6): 1629-1636.

Sangamwar, A. T.; Deshpande, U. D. and Pekamwar, S. S. (2008). Antifungals: Need to search for a new molecular target. *Indian Journal of Pharmaceutical Sciences*. 70(4): 423-430.

Saravolatz, L. D.; Deresinski, S. C. and Stevens, D. A. (2003). Caspofungin. *Clinical Infectious Diseases*. 36(11): 1445-1457.

Schagerl, M.; Unterrieder, I. and Angeler, D. G. (2002). Allelopathy among cyanoprokaryota and other algae originating from Lake Neusiedlersee (Austria). *International Review of Hydrobiology*. 87(4): 365-374.

Schindle, D. W. (1977). Evolution and phosphorus limitation in lakes. *Science*. 195: 260-262.

Schlegel, I.; Doan, N.; de Chazal, N. and Smith, G. (1998). Antibiotic activity of new cyanobacterial isolates from Australia and Asia against green algae and cyanobacteria. *Journal of Applied Phycology*. 10(5): 471-479.

Seki, H.; Ozawa, H. and Ichimura, S.-e. (1981). Temperature dependence of filament length of *Anabaena spiroides* Klebahn var. crassa Lemm. *Hydrobiologia*. 83(3): 419-423.

Shalini, K.; Kumar, N.; Drabu, S. and Sharma, P. K. (2011). Advances in synthetic approach to and antifungal activity of triazoles. *Beilstein Journal of Organic Chemistry*. 7: 668-677.

Shanab, M. M. S. (2007). Bioactive allelo-chemical compounds from *Oscillatoria* species (Egyptian isolates) *International Journal of Agriculture & Biology*. 9(4).

Shanab, S. M. M.; Saker, M. M. and Abdel-Rahman, M. H. M. (2003). Crude extracts of some fresh water cyanobacteria have auxinlike activity on potato tissue culture. *Arab Journal of Biotechnology*. 6(2): 297-312.

Shao, P.-L.; Huang, L.-M. and Hsueh, P.-R. (2007). Recent advances and challenges in the treatment of invasive fungal infections. *International Journal of Antimicrobial Agents*. 30(6): 487-495.

Sharma, N.; Tiwari, S.; Tripathi, K. and Rai, A. (2011). Sustainability and cyanobacteria (blue-green algae): facts and challenges. *Journal of Applied Phycology*. 23(6): 1059-1081.

Sharma, V.; Kumar, P. and Pathak, D. (2010). Biological importance of the indole nucleus in recent years: A comprehensive review. *Journal of Heterocyclic Chemistry*. 47.

Shin, H. J.; Murakami, M.; Matsuda, H. and Yamaguchi, K. (1996). Microviridins D-F, serine protease inhibitors from the cyanobacterium *Oscillatoria agardhii* (NIES-204). *Tetrahedron*. 52(24): 8159-8168.

Shin, H. J.; Matsuda, H.; Murakami, M. and Yamaguchi, K. (1997a). Aeruginosins 205A and -B, serine protease inhibitory glycopeptides from the cyanobacterium *Oscillatoria agardhii* (NIES-205). *The Journal of Organic Chemistry*. 62(6): 1810-1813.

(1997b). Circinamide, a novel papain inhibitor from the cyanobacterium *Anabaena circinalis* (NIES-41). *Tetrahedron*. 53(16): 5747-5754.

Silva, P. C.; Meñez, E. G. and Moe, R. L. (1987). Catalog of the benthic marine algae of the Philippines. Issues 27-28. Washington, D. C., *Smithsonian Institution Press*.

Sim, A. T. R. and Mudge, L.-M. (1993). Protein phosphatase activity in cyanobacteria: Consequences for microcystin toxicity analysis. *Toxicon*. 31(9): 1179-1186.

Singh, I. P.; Milligan, K. E. and Gerwick, W. H. (1999). Tanikolide, a toxic and antifungal lactone from the marine cyanobacterium *Lyngbya majuscula*. *Journal of Natural Products*. 62(9): 1333-1335.

Singh, S. (1989). Cobalt-induced inhibition of growth in cyanobacteria *Anabaena doliolum* and *Anacystis nidulans*: interaction with sulphur containing amino acids. *Indian Journal of Experimental Biology*. 27(12): 1092-1093.

Singh, S.; Kate, B. N. and Banerjee, U. C. (2005). Bioactive compounds from cyanobacteria and microalgae: An overview. *Critical Reviews in Biotechnology*. 25(3): 73-95.

Singhal, T. (2013). Newer antifungals. *Pediatric Infectious Disease*. 5(2): 78-82.

Sivonen, K.; Kononen, K.; Carmichael, W. W.; Dahlem, A. M.; Rinehart, K. L.; Kiviranta, J. and Niemela, S. I. (1989a). Occurrence of the hepatotoxic cyanobacterium *Nodularia spumigena* in the Baltic Sea and structure of the toxin. *Applied Environmental Microbiology*. 55(8): 1990-1995.

Sivonen, K.; Kononen, K.; Esala, A. L. and Niemelä, S. I. (1989b). Toxicity and isolation of the cyanobacterium *Nodularia spumigena* from the southern Baltic Sea in 1986. *Hydrobiologia*. 185(1): 3-8.

Sivonen, K. (1990). Effects of light, temperature, nitrate, orthophosphate, and bacteria on growth of and hepatotoxin production by *Oscillatoria agardhii* strains. *Appiled and Environmental Microbiology*. 56(9): 2658–2666.

Sivonen, K.; Halinen, K.; Sihvonen, L. M.; Koskenniemi, K.; Sinkko, H.; Rantasärkkä, K.; Moisander, P. H. and Lyra, C. (2007). Bacterial diversity and function in the Baltic Sea with an emphasis on cyanobacteria. *AMBIO: A Journal of the Human Environment*. 36(2): 180-185.

Sivonen, K.; Leikoski, N.; Fewer, D. and Jokela, J. (2010). Cyanobactins - ribosomal cyclic peptides produced by cyanobacteria. *Applied Microbiology and Biotechnology*. 86(5): 1213-1225.

Smith, D. C.; Carmeli, S. and Moore, E. R. (1993). Scytophycins, novel microfilament-depolymerizing agents which circumvent p-glycoprotein-mediated multidrug resistance. *Cancer Research*. 53: 1343-1347.

Smith, V. H. (1983). Low nitrogen to phosphorus ratios favor dominance by blue-green algae in lake phytoplankton. *Science*. 221: 669-671.

Soltani, N.; Khavari-Nejad, R. A.; Yazdi, M. T.; Shokravi, S. and Fernandez-Valiente, E. (2005). Screening of soil cyanobacteria for antifungal and antibacterial activity. *Pharmaceutical Biology*. 43(5): 455–459.

Srivastava, V. C.; Manderson, G. J. and Bhamidimarri, R. (1999). Inhibitory metabolites production by the cyanobacterium *Fischerella muscicola*. *Microbiological Research*. 153(4): 309-317.

Stal, L. J.; Albertano, P.; Bergman, B.; Bröckel, K. v.; Gallon, J. R.; Hayes, P. K.; Sivonen, K. and Walsby, A. E. (2003). BASIC: Baltic Sea cyanobacteria. An investigation of the structure and dynamics of water blooms of cyanobacteria in the Baltic Sea—responses to a changing environment. *Continental Shelf Research*. 23(17–19): 1695-1714.

Stamatakis, A. (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*. 22(21): 2688-2690.

Stanier, R. Y.; Kunisawa, R.; Mandel, M. and Cohen-Bazire, G. (1971). Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriological Reviews*. 35(2): 171-205.

Stanier, R. Y.; Sistrom, W. R.; Hansen, T. A.; Whitton, B. A.; Castenholz, R. W.; Pfennig, N.; Gorlenko, V. N.; Kondratieva, E. N.; Eimhjellen, K. E.; Whittenbury, R.; Gherna, R. L. and Truper, H. G. (1978). Proposal to place the nomenclature of the cyanobacteria (blue-green algae) under the rules of the international code of nomenclature of bacteria. *International Journal of Systematic Bacteriology*. 28(2): 335-336.

Stewart, I.; Schluter, P. J. and Shaw, G. R. (2006). Cyanobacterial lipopolysaccharides and human health – a review. 6(7).

Stewart, J. B.; Bornemann, V.; Chen, L. J.; Moore, E. R.; Caplan, R. F.; Karuso, H.; Larsen, K. L. and Patterson, G. M. L. (1988). Cytotoxic, fungicidal nucleosides from blue green algae belong to the Scytonemataceae. *The Journal of Antibiotics*. 41(8): 1048-1056.

Stratmann, K.; Moore, R. E.; Bonjouklian, R.; Deeter, J. B.; Patterson, G. M. L.; Shaffer, S.; Smith, C. D. and Smitka, T. A. (1994). Welwitindolinones, unusual alkaloids from the blue-green algae *Hapalosiphon welwitschii* and *Westiella intricata*. Relationship to fischerindoles and hapalinodoles. *Journal of the American Chemical Society*. 116(22): 9935-9942.

Suárez-Castillo, O. R.; Sánchez-Zavala, M.; Meléndez-Rodríguez, M.; Castelán-Duarte, L. E.; Morales-Ríos, M. S. and Joseph-Nathan, P. (2006). Preparation of 3-hydroxyoxindoles with dimethyldioxirane and their use for the synthesis of natural products. *Tetrahedron*. 62(13): 3040-3051.

Sucher, A. J.; Chahine, E. B. and Balcer, H. E. (2009). Echinocandins: the newest class of antifungals. *The Annals of Pharmacotherapy*. 43(10): 1647-1657.

Suffness, M. and Pezzuto, J. M. (1991). Assays related to cancer drug discovery. Methods in Plant Biochemistry. Hostettmann K. London: *Academic Press*. 6: 71-133.

Suikkanen, S.; Fistarol, O. G. and Grane´li, E. (2004). Allelopathic effects of the Baltic cyanobacteria *Nodularia spumigena*, *Aphanizomenon flos-aquae* and *Anabaena lemmermannii* on algal monocultures. *Journal of Experimental Marine Biology and Ecology*. 308: 85 - 101.

Suikkanen, S.; Engström-Öst, J.; Jokela, J.; Sivonen, K. and Viitasalo, M. (2006). Allelopathy of Baltic Sea cyanobacteria: no evidence for the role of nodularin. *Journal of Plankton Research*. 28(6): 543-550.

Suikkanen, S. (2008). Allelopathic effects of filamentous cyanobacteria on phytoplankton in the Baltic Sea. *University of Helsinki, Finland*. Dissertation.

Sunda, W. G. and Huntsman, S. A. (1995). Cobalt and Zinc interreplacement in marine phytoplankton: Biological and geolchemical implications. *Limnology and Ocenaography*. 40(8): 1404-1417.

Surakka, A.; Sihvonen, L. M.; Lehtimäki, J. M.; Wahlsten, M.; Vuorela, P. and Sivonen, K. (2005). Benthic cyanobacteria from the Baltic Sea contain cytotoxic *Anabaena*, *Nodularia*, and *Nostoc* strains and an apoptosis-inducing *Phormidium* strain. *Environmental Toxicology*. 20(3): 285-292.

Svircev, Z.; Cetojevic-Simin, D.; Simeunovic, J.; Karaman, M. and Stojanovic, D. (2008). Antibacterial, antifungal and cytotoxic activity of terrestrial cyanobacterial strains from Serbia. *Science in China Series C: Life Sciences*. 51(10): 941-947.

Tadera, K. and Orite, K. (1991). Isolation and Structure of a New Vitamin B_6 Conjugate in Rice Bran. *Journal of Food Science*. 56(1): 268-269.

Tamas, I.; Svircev, Z. and Andersson, S. G. E. (2000). Determinative value of a portion of the *nif*H sequence for the genera *Nostoc* and *Anabaena* (cyanobacteria). *Current Microbiology*. 41(3): 197-200.

Tan, L. T. (2007). Bioactive natural products from marine cyanobacteria for drug discovery. *Phytochemistry*. 68(7): 954-979.

Tanabe, Y.; Kasai, F. and Watanabe, M. M. (2007). Multilocus sequence typing (MLST) reveals high genetic diversity and clonal population structure of the toxic cyanobacterium *Microcystis aeruginosa*. *Microbiology*. 153(11): 3695-3703.

Tang, E. P. Y.; Tremblay, R. and Vincent, W. F. (1997). Cyanobacterial dominance of polar freshwater ecosystems: Are high-latitude mat-formers adapted to low temperature? *Journal of Phycology*. 33(2): 171-181.

Tellez, M.; Estell, R.; Fredrickson, E.; Powell, J.; Wedge, D.; Schrader, K. and Kobaisy, M. (2001). Extracts of *Flourensia cernua* (L): Volatile constituents and antifungal, antialgal, and antitermite bioactivities. *Journal of Chemical Ecology*. 27(11): 2263-2273.

Teuscher, E., Lindequist, U., Mundt, S. (1992). Cyanobakterien, Quellen biogener Wirkstoffe. . *Pharm. Ztg. Wiss.* 137: 57-69.

Thornburg, C. C.; Thimmaiah, M.; Shaala, L. A.; Hau, A. M.; Malmo, J. M.; Ishmael, J. E.; Youssef, D. T. A. and McPhail, K. L. (2011). Cyclic depsipeptides, grassypeptolides D and E and ibu-epidemethoxylyngbyastatin 3, from a Red Sea *Leptolyngbya* cyanobacterium. *Journal of Natural Products*. 74: 1677–1685.

Tillett, D.; Dittmann, E.; Erhard, M.; von Döhren, H.; Börner, T. and Neilan, B. A. (2000). Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide polyketide synthetase system. *Chemistry & biology*. 7(10): 753-764.

Tiwari, O. N.; Singh, B. V.; Mishra, U.; Singh, A. K.; Dhar, D. W. and Singh, P. K. (2005). Distribution and physiological characterization of cyanobacteria isolated from arid zones of Rajasthan. *Tropical Ecology*. 46(2): 165-171.

Tonk, L.; Visser, P. M.; Christiansen, G.; Dittmann, E.; Snelder, O. F. M. E.; Wiedner, C.; Mur, L. R. and Huisman, J. (2005). The microcystin composition of the cyanobacterium *Planktothrix agardhii* changes toward a more toxic variant with increasing light intensity. *Applied and Environmental Microbiology*. 71(9): 5177-5181.

Tooming-Klunderud, A.; Rohrlack, T.; Shalchian-Tabrizi, K.; Kristensen, T. and Jakobsen, K. S. (2007). Structural analysis of a non-ribosomal halogenated cyclic peptide and its putative operon from *Microcystis*: implications for evolution of cyanopeptolins. *Microbiology*. 153(5): 1382-1393.

Trimurtulu, G.; Ohtani, I.; Patterson, G. M. L.; Moore, R. E.; Corbett, T. H.; Valeriote, F. A. and Demchik, L. (1994). Total structures of cryptophycins, potent antitumor depsipeptides from the blue-green alga *Nostoc* sp. strain GSV 224. *Journal of the American Chemical Society*. 116(11): 4729-4737.

Uehlinger, U. (1981). Experimental studies of the autecology of *Aphanizomenon flos-aquae*. *Archiv für Hydrobiologie*. Supplement 60: 260-288.

US FDA (2001). Guidance for industry-bioanalytical method validation. Bioanalytical Method Validation.

Utkilen, H. and Gjolme, N. (1992). Toxin production by *Microcystis aeruginosa* as a function of light in continuous cultures and its ecological significance. *Applied and Environmental Microbiology*. 58(4): 1321-1325.

Utkilen, H.; Mur, R. L.; Skulberg, M. O. and Utkilen, H. (1999). Cyanobacteria in the environment. Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management. Chorus I. and Bartram J.

Vaccarino, M. A.; Tillmaand, E. G.; Martin, M. P. and Johansen, J. R. (2009). Phylogenetic placement of the Microchaetaceae (Cyanobacteria). Plant Biology 2009. Honolulu, Hawaii, *American Society of Plant Biologists*.

Vaishampayan, U.; Glode, M.; Du, W.; Kraft, A.; Hudes, G.; Wright, J. and Hussain, M. (2000). Phase II study of dolastatin-10 in patients with hormone-refractory metastatic prostate adenocarcinoma. *Clinical Cancer Research*. 6(11): 4205-4208.

Van Apeldoorn, M. E.; Van Egmond, H. P.; Speijers, G. J. A. and Bakker, G. J. I. (2007). Toxins of cyanobacteria. *Molecular Nutrition & Food Research*. 51(1): 7-60.

van der Westhuizen, A. J. and Eloff, J. N. (1983). Effect of culture age and pH of culture medium on the growth and toxicity of the blue-green alga *Microcystis aeruginosa*. *Zeitschrift für Pflanzenphysiologie*. 110(2): 157-163.

Vasas, G.; Borbely, G.; Nánási, P. and Nánási, P. P. (2010). Alkaloids from cyanobacteria with diverse powerful bioactivities. *Mini Reviews in Medicinal Chemistry*. 10(10): 946-955.

Venkataraman, L. V. (1997). *Spirulina platensis* (*Arthrospira*): Physiology, cell biology and biotechnologym. *Journal of Applied Phycology*. 9(3): 295-296.

Vézie, C.; Rapala, J.; Vaitomaa, J.; Seitsonen, J. and Sivonen, K. (2002). Effect of nitrogen and phosphorus on growth of toxic and nontoxic *Microcystis* strains and on intracellular microcystin concentrations. *Microbial Ecology*. 43(4): 443-454.

Vla, K. (1980). Reactions of cyanobacteria to certain heavy metals. *Mikrobiologiia*. 49(5): 821-824.

Vincent, W. F. and Silvester, W. B. (1979). Growth of bluegreen algae in the Manukau (New Zealand) oxidation ponds. I. Growth potential of oxidation pond water and comparative optima for blue-green and green algal growth. *Water research*. 13: 711-716.

Vincent, W. F. (1989). Cyanobacterial growth and dominance in two eutrophic lakes: review and synthesis. *Hydrobiologie–Beiheft Ergebnisse der Limnologie*. 32: 239-254.

Volk, R.-B. (2005). Screening of microalgal culture media for the presence of algicidal compounds and isolation and identification of two bioactive metabolites, excreted by the cyanobacteria *Nostoc insulare* and *Nodularia harveyana*. *Journal of Applied Phycology*. 17(4): 339-347.

Volk, R.-B. and Furkert, F. H. (2006). Antialgal, antibacterial and antifungal activity of two metabolites produced and excreted by cyanobacteria during growth. *Microbiological Research*. 161(2): 180-186.

Wada, H.; Schmidt, H.; Heinz, E. and Murata, N. (1993). *in vitro* ferredoxin-dependent desaturation of fatty acids in cyanobacterial thylakoid membranes. *Journal of Bacteriology*. 175(2): 544-547.

Wasmund, N. (1997). Occurrence of cyanobacterial blooms in the Baltic Sea in relation to environmental conditions. *Internationale Revue der gesamten Hydrobiologie und Hydrographie*. 82(2): 169-184.

Watanabe, F.; Tanioka, Y.; Miyamoto, E.; Fujita, T.; Takenaka, H. and Nakano, Y. (2007). Purification and characterization of corrinoid-compounds from the dried powder of an edible cyanobacterium, *Nostoc commune* (Ishikurage). *Journal of Nutritional Science and Vitaminology*. 53(2): 183-186.

Watanabe, M.; Harada, K.-I.; Matsuura, K.; Watanabe, M. and Suzuki, M. (1989). Heptapeptide toxin production during the batch culture of two *Microcystis* species (Cyanobacteria). *Journal of Applied Phycology*. 1(2): 161-165.

Watanabe, M. F. and Oishi, S. (1985). Effects of environmental factors on toxicity of a cyanobacterium (*Microcystis aeruginosa*) under culture conditions. *Applied and Environmental Microbiology*. 49(5): 1342-1344.

Waterbury, J. B. (1989). Order Pleurocapsales Geitler 1925, emend. Waterbury and Stanier 1978. Bergey's manual of systematic bacteriology, vol. 3. Staley J. T., Bryant M. P., Pfennig N. and Holts J. G. Baltimore: *Williams and Wilkins*. 3: 1746–1770.

Waterbury, J. B. and Rippka, R. (1989). The order Chroococcales Wettstein 1924, emend. Rippka et al., 1979. Bergey's manual of systematic bacteriology, vol. 3. Staley J. T., Bryant M. P., Pfennig N. and Holts J. G. Baltimore: *Williams and Wilkins*. 3: 1728–1746.

Waterbury, J. B. (2006). The cyanobacteria—isolation, purification and identification. *Prokaryotes*. 4: 1053-1073.

Welker, M. and Von Döhren, H. (2006). Cyanobacterial peptides – Nature's own combinatorial biosynthesis. *FEMS Microbiology Reviews*. 30(4): 530-563.

Westhuizen, A. J. and Eloff, J. N. (1985). Effect of temperature and light on the toxicity and growth of the blue-green alga *Microcystis aeruginosa* (UV-006). *Planta*. 163(1): 55-59.

Wiedner, C.; Visser, M. P.; Fastner, J.; Metcalf, S. J.; Codd, A. G. and Mur, R. L. (2003). Effects of light on the microcystin content of *Microcystis* strain PCC 7806. *Applied and Environmental Microbiology*. 69(3): 1475-1481.

Wiese, M.; D'Agostino, P. M.; Mihali, T. K.; Moffitt, M. C. and Neilan, B. A. (2010). Neurotoxic alkaloids: Saxitoxin and its analogs. *Marine Drugs*. 8(2185-2211).

Williams, P. G.; Yoshida, W. Y.; Moore, R. E. and Paul, V. J. (2001). Isolation and structure determination of obyanamide, a novel cytotoxic cyclic depsipeptide from the marine cyanobacterium *Lyngbya confervoides*. *Journal of Natural Products*. 65(1): 29-31.

(2004). Micromide and guamamide: cytotoxic alkaloids from a species of the marine *Cyanobacterium symploca*. *Journal of Natural Products*. 67(1): 49-53.

Wu, M.; Okino, T.; Nogle, L. M.; Marquez, B. L.; Williamson, R. T.; Sitachitta, N.; Berman, F. W.; Murray, T. F.; McGough, K.; Jacobs, R.; Colsen, K.; Asano, T.; Yokokawa, F.; Shioiri, T. and Gerwick, W. H. (2000). Structure, synthesis, and biological properties of kalkitoxin, a novel neurotoxin from the marine cyanobacterium *Lyngbya majuscula*. *Journal of the American Chemical Society*. 122(48): 12041-12042.

Yamazaki, T.; Kume, H.; Murase, S.; Yamashita, E. and Arisawa, M. (1999). Epidemiology of visceral mycoses: Analysis of data in annual of the pathological autopsy cases in Japan. *Journal of Clinical Microbiology*. 37(6): 1732-1738.

Yu, D. T.; Peterson, J. F.; Seger, D. L.; Gerth, W. C. and Bates, D. W. (2005). Frequency of potential azole drug–drug interactions and consequences of potential fluconazole drug interactions. *Pharmacoepidemiology and Drug Safety*. 14(11): 755-767.

Zaccaroni, A. and Scaravelli, D. (2008). Toxicity of fresh water algal toxins to humans and animals algal toxins: Nature, occurrence, effect and detection. Evangelista V., Barsanti L., Frassanito A. M., Passarelli V. and Gualtieri P., *Springer Netherlands*. 45-89.

Zainuddin, E. N.; Jansen, R.; Nimtz, M.; Wray, V.; Preisitsch, M.; Lalk, M. and Mundt, S. (2009). Lyngbyazothrins A-D, antimicrobial cyclic undecapeptides from the cultured cyanobacterium *Lyngbya* sp. *Journal of Natural Products*. 72: 1373–1378.

Zainuddin, E. Z.; Mundt, S. M.; Wegner, U. W. and Mentel, R. M. (2002). Cyanobacteria a potential source of antiviral substances against influenza virus. *Medical Microbiology and Immunology*. 191(3): 181-182.

Zorica Svircev, D. C.-S., Jelica Simeunovic, Maja Karaman, Dejan Stojanovic (2008). Antibacterial, antifungal and cytotoxic activity of terrestrial cyanobacterial strains from Serbia. *Science in China Series C: Life Sciences*. 51(10): 941-947.

Appendix

I. Antifungal assay

Protokoll 09.11.2009

Testung von Cyanobacterienextrakten auf antimykotische Wirksamkeit

Methode: Agardiffusionstest (nach CLSI)

Testmedium: RPMI-Agar, Fa. AB Biodisk

(Synthetisches Medium zur Antimykotika-Testung nach NCCLS-Standard)

Teststämme:

Spezies	Pilzgruppe	Herkunft
Candida albicans ATCC 90028	Sprosspilz	Stammsammlung
Candida krusei ATCC 90878	Sprosspilz	Stammsammlung
Aspergillus fumigatus	Schimmelpilz	Patientenisolat
Mucor sp.	Schimmelpilz (Klasse: Zygomycetes)	Patientenisolat
Microsporum gypseum	Dermatophyt	INSTAND Ringversuch, 2009

Ergebnisse:

	Spezies				
	C. albicans	*C. krusei*	*A. fumigatus*	*Mucor sp.*	*M. gypseum*
Bebrütungszeit/-temperatur	24h/36°C	24h/36°C	48h/36°C	48h/30°C	7d/25°C
Substanz-Nr.	HHD/mm	HHD/mm	HHD/mm	HHD/mm	HHD/mm
1	0	0	0	0	0
2	23	20	30	26	38
Nystatin Kontrolle	24	19	24	25	20

1: MeOH extract of TVN40; 2: FIII of Bio 33

Protokoll 10.09.2010

Testung von Cyanobakterienextrakteextrakten auf antimykotische Wirksamkeit

Methode: Agardiffusionstest (nach CLSI)

Testmedium: RPMI-Agar, Fa. AB Biodisk. (Synthetisches Medium zur Antimykotika-Testung nach CLSI-Standard)

Dr. Gudrun Schröder. Institut für Medizinische Mikrobiologie

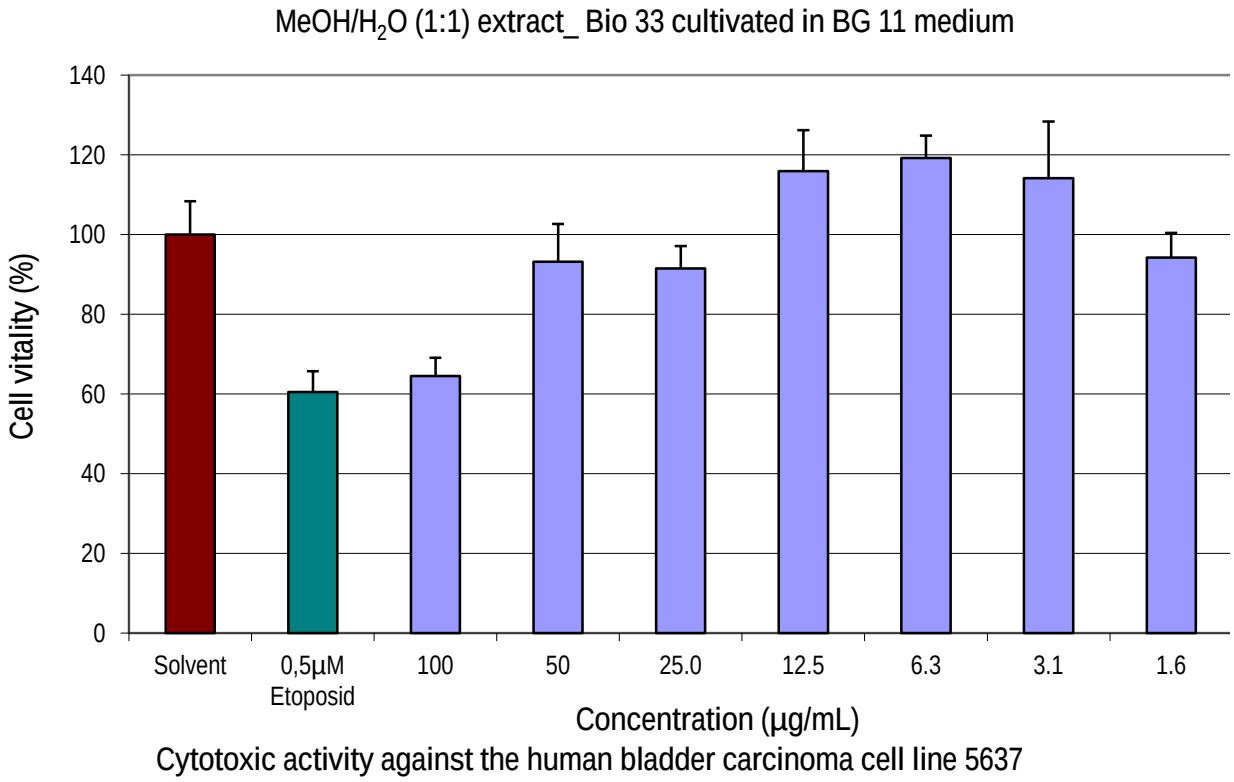
Teststämme:

Spezies	Pilzgruppe	Herkunft
Candida albicans ATCC 90028	Sprosspilz	Stammsammlung
Candida krusei ATCC 90878	Sprosspilz	Stammsammlung
Aspergillus fumigatus	Schimmelpilz	Patientenisolat
Mucor sp.	Schimmelpilz (Klasse: Zygomycetes)	Stammsammlung
Trichophyton rubrum	Dermatophyt	INSTAND Ringversuch, 2008

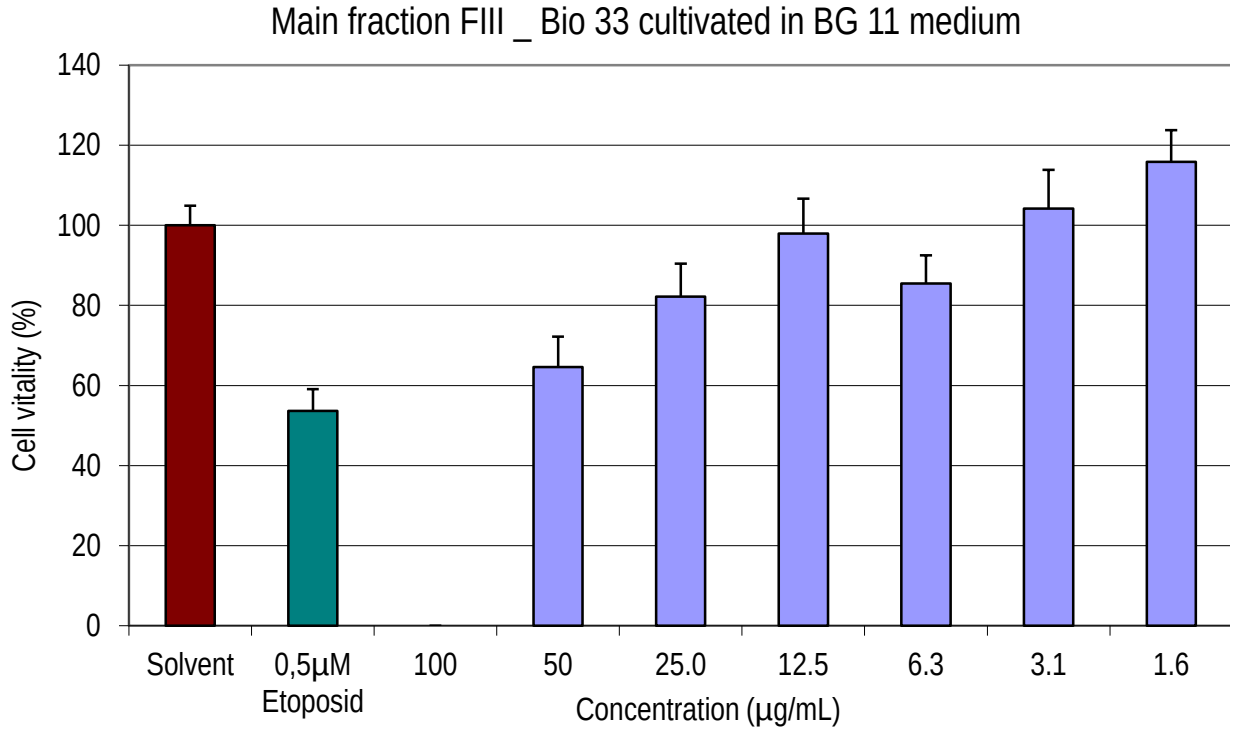
Ergebnisse:

		Spezies									
		C. albicans		*C. krusei*		*A. fumigatus*		*Mucor sp.*		*T. rubrum*	
Bebrütungszeit/-temperatur		24h/36°C		24h/36°C		48h/36°C		48h/30°C		7d/25°C	
Substanz-Nr.		HHD/mm		HHD/mm		HHD/mm		HHD/mm		HHD/mm	
		1.	2.	1.	2.	1.	2.	1.	2.		
1	1	0	0	0	0	0	0	10			
2	233	0	0	0	0	0	0	0			
3	1611	0	0	0	0	0	0	0			
4	AS	0	0	0	0	0	0	0			
5	154	0	0	0	0	0	0	0			
6	Asb	0	0	0	0	0	0	0			
7	Bio 33 - axenic	16	16	13	12	25	26	25			
Nystatin Kontrolle	Nystatin Control	23		21		23	24				

II. Cytotoxic assay



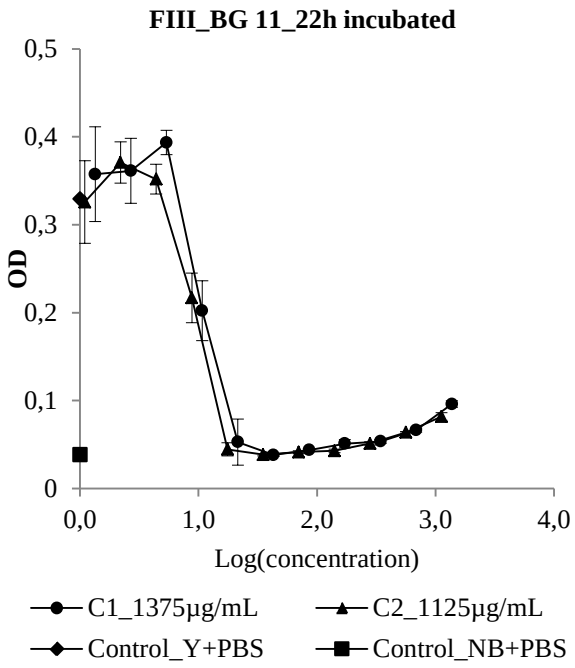
Cytotoxic activity against the human bladder carcinoma cell line 5637



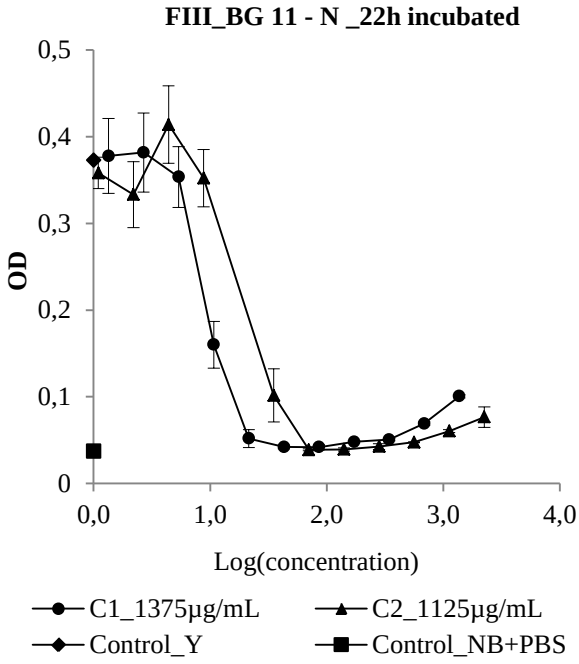
Cytotoxic activity against the human bladder carcinoma cell line 5637

III. MIC of the main fraction FIII

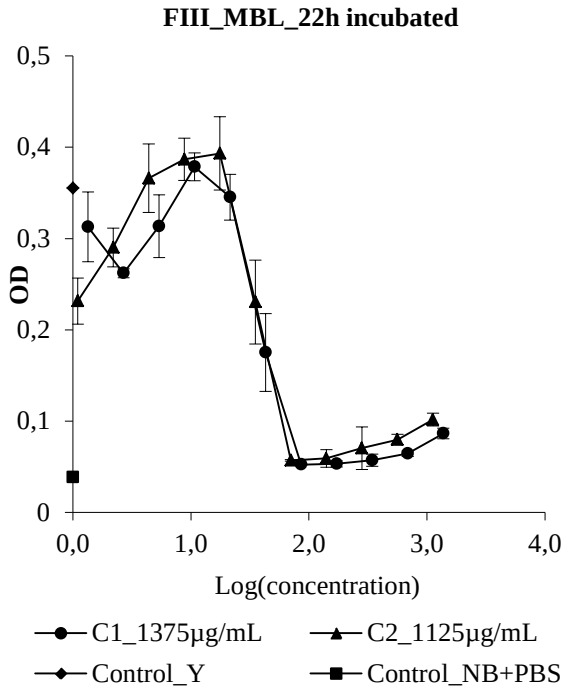
a)



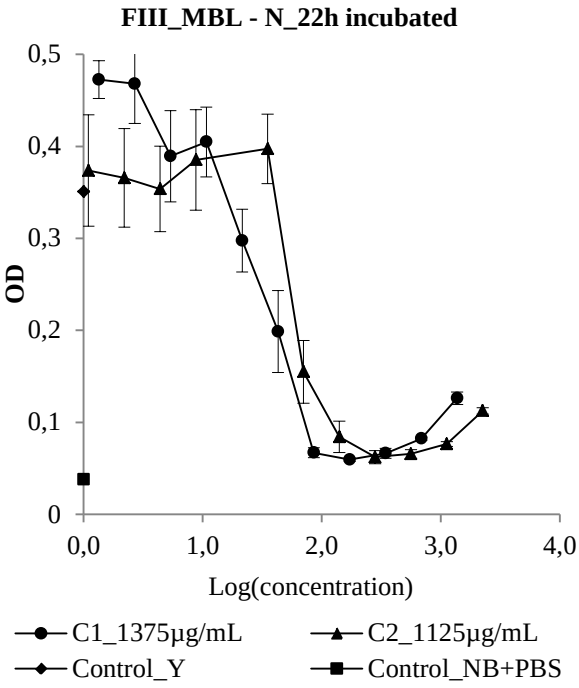
b)



c)



d)



Estimation of MIC for the main fraction FIII of strain Bio 33 cultivated in different media. a) BG 11. b) BG 11 without $NaNO_3$. c) MBL. d) MBL without $NaNO_3$. Control_Y: PBS + Bouillon + *C.m*. Control_NB+PBS: PBS + Bouillon. n = 4 parallels.

IV. Supplementary information of structural elucidation of balticidins 1-4

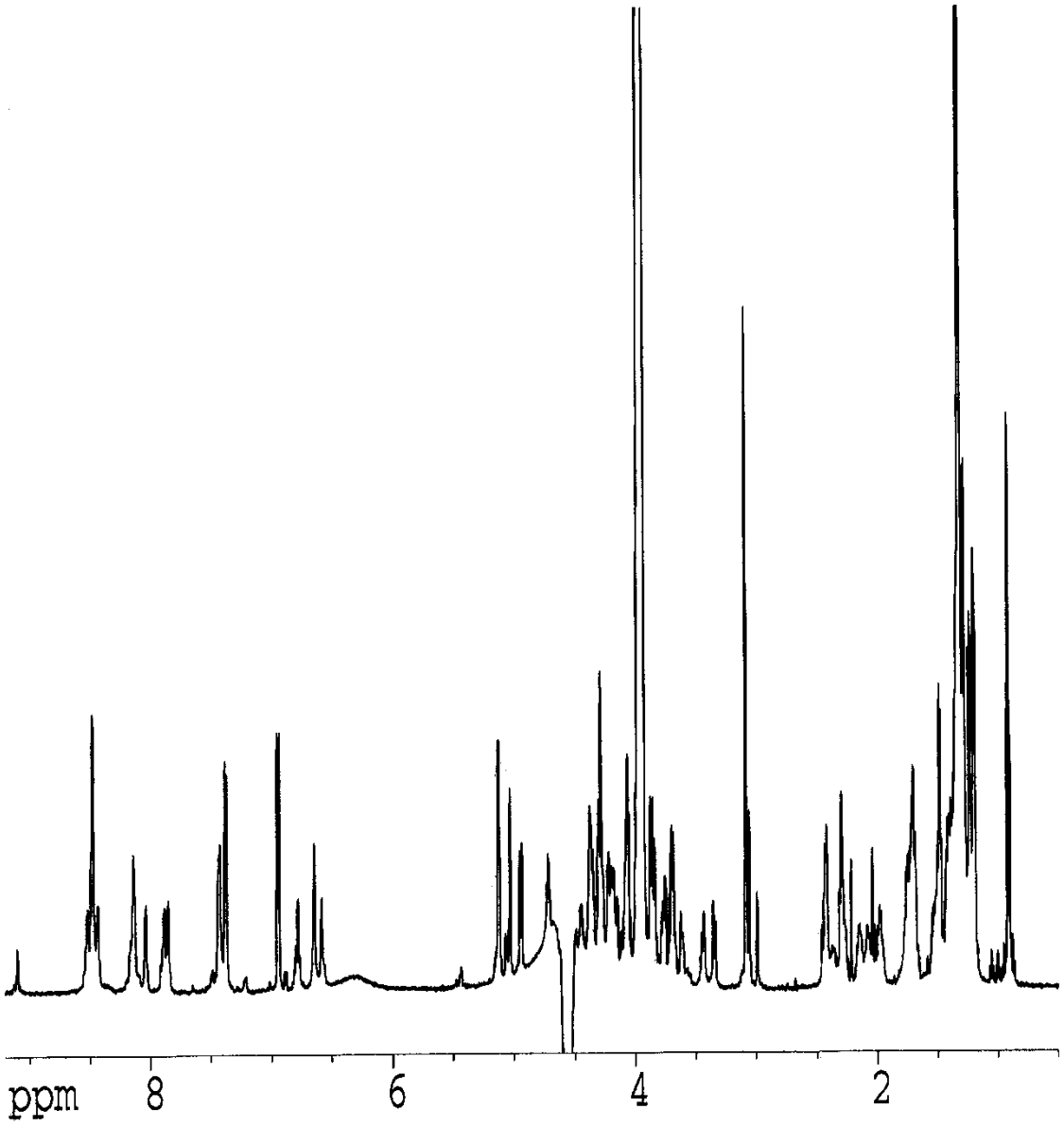


Figure 1S1. 1D ^{1}H NMR spectrum of 1 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

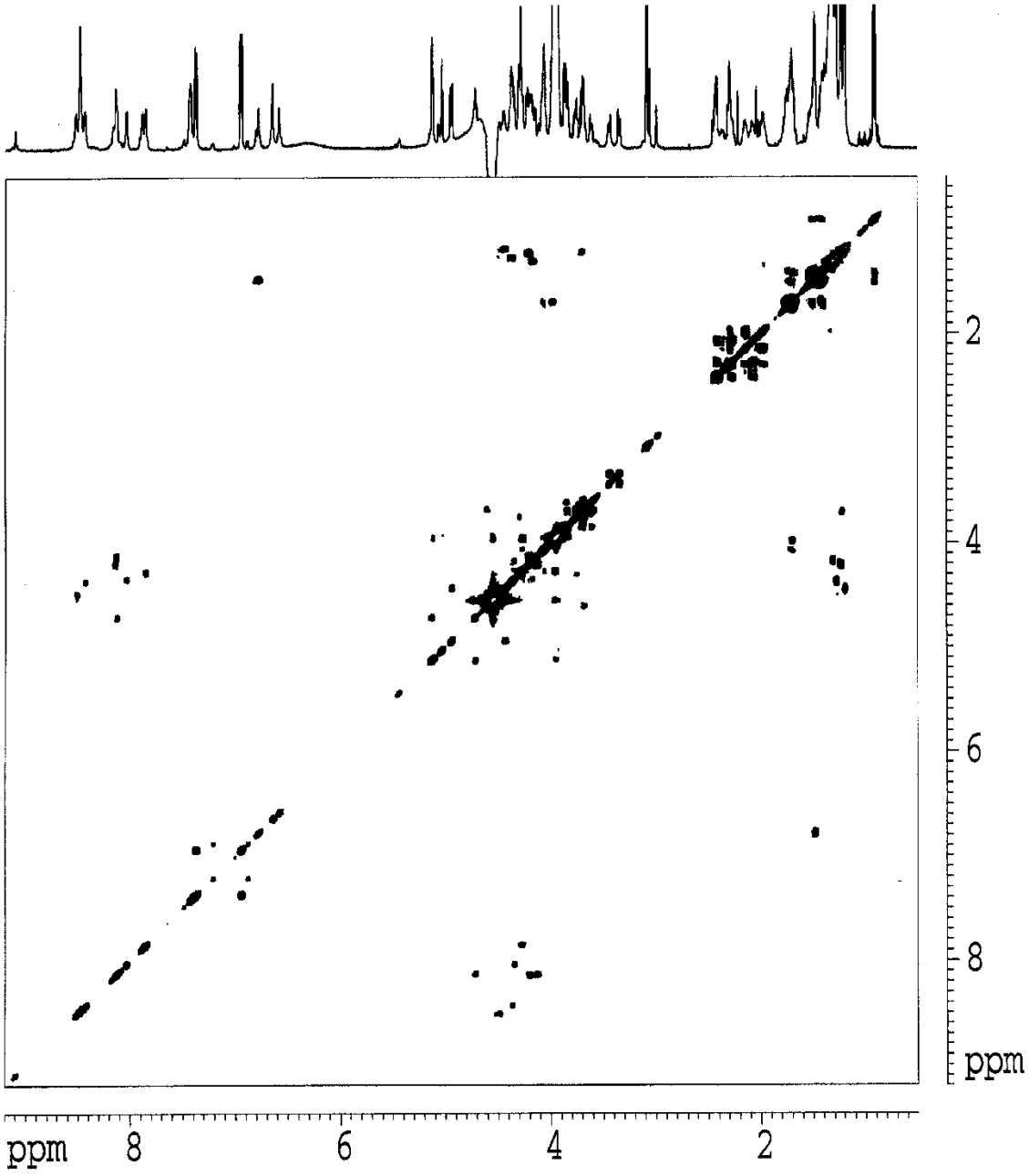


Figure 1S2. 2D COSY spectrum of 1 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

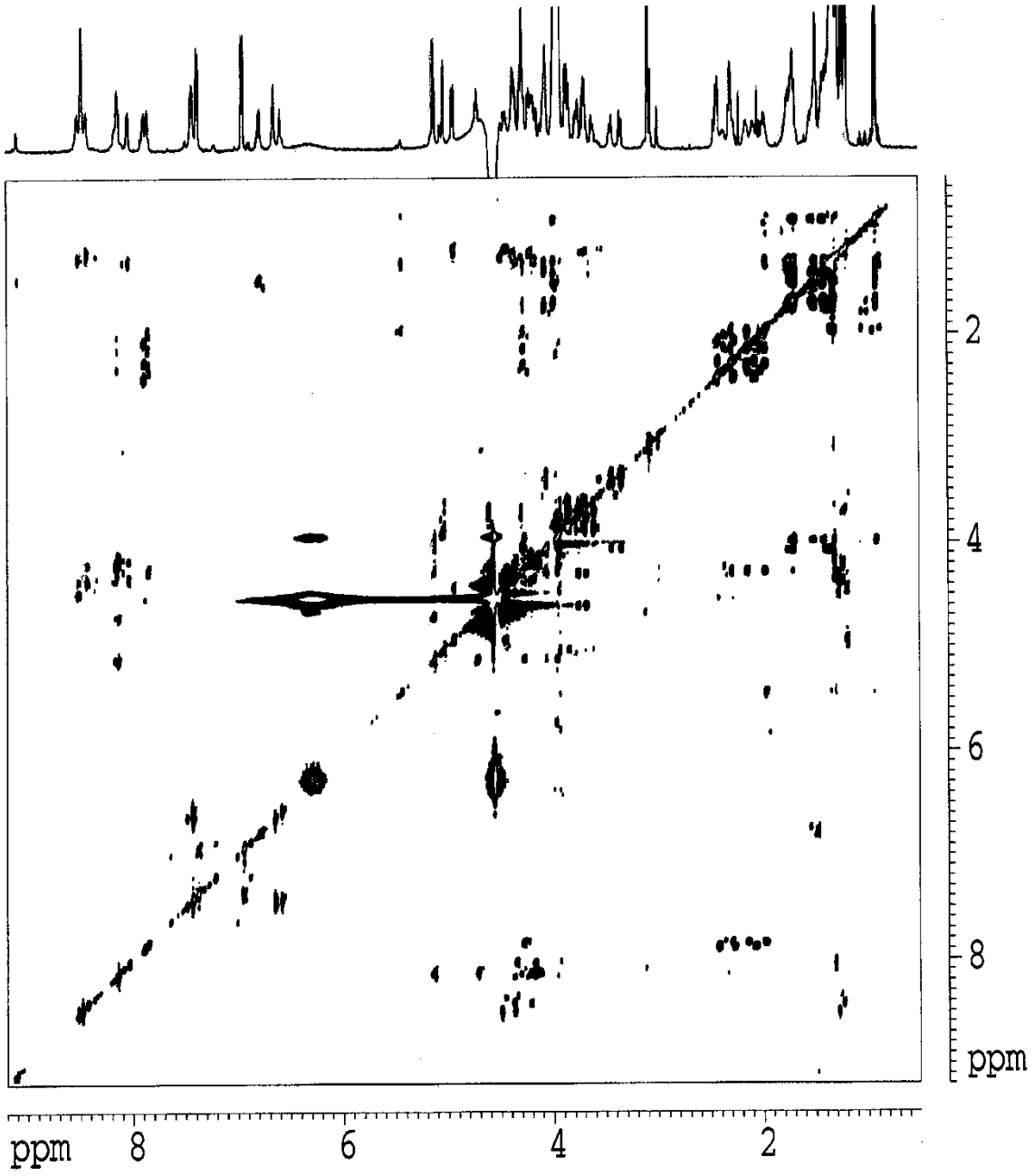


Figure 1S3. 2D TOCSY spectrum of 1 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

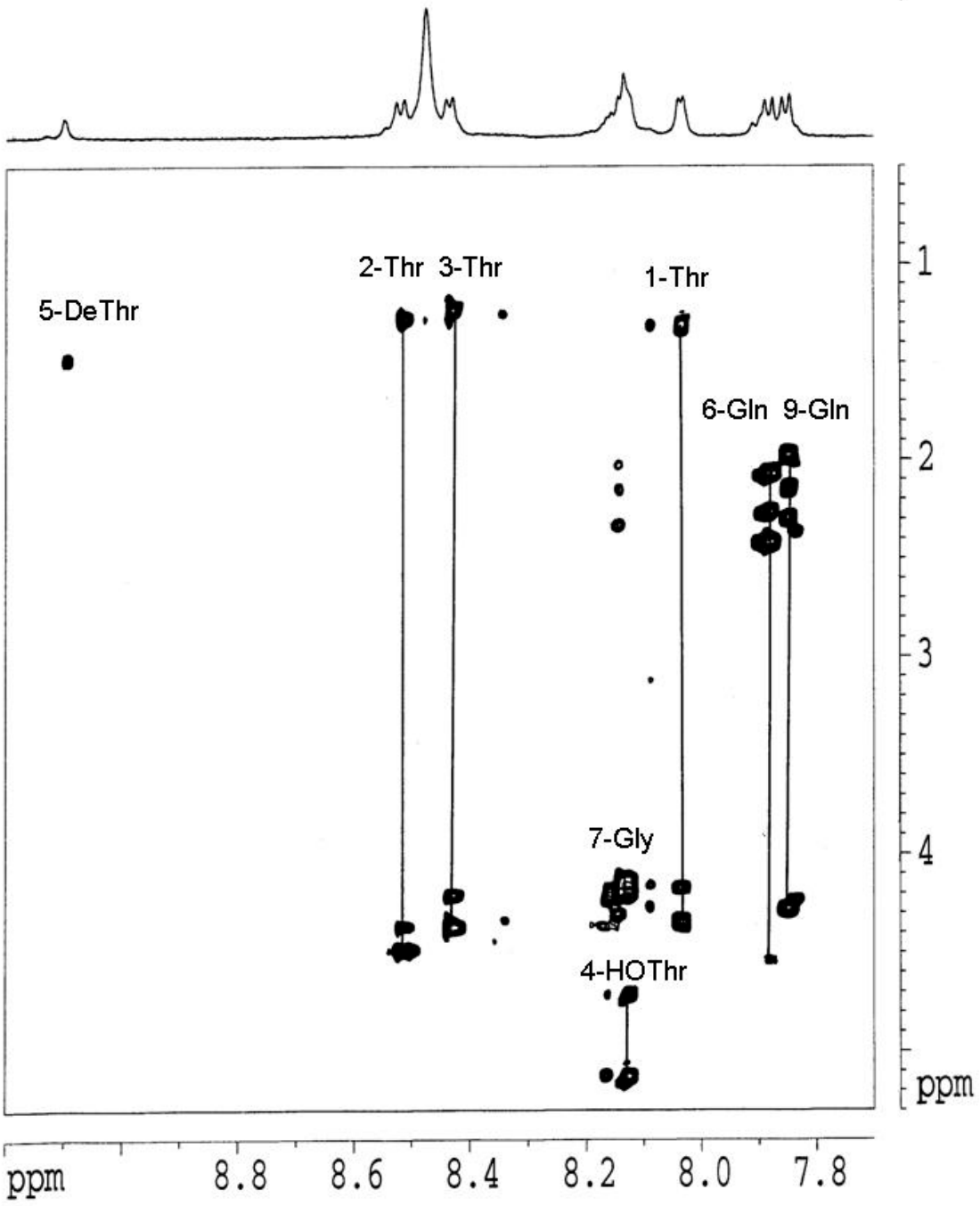


Figure 1S4. Low-field region of the 2D TOCSY spectrum of 1 in trifluoroethanol-d_2/H_2O (1:1) at 300 K showing assignments of amino acid spin systems.

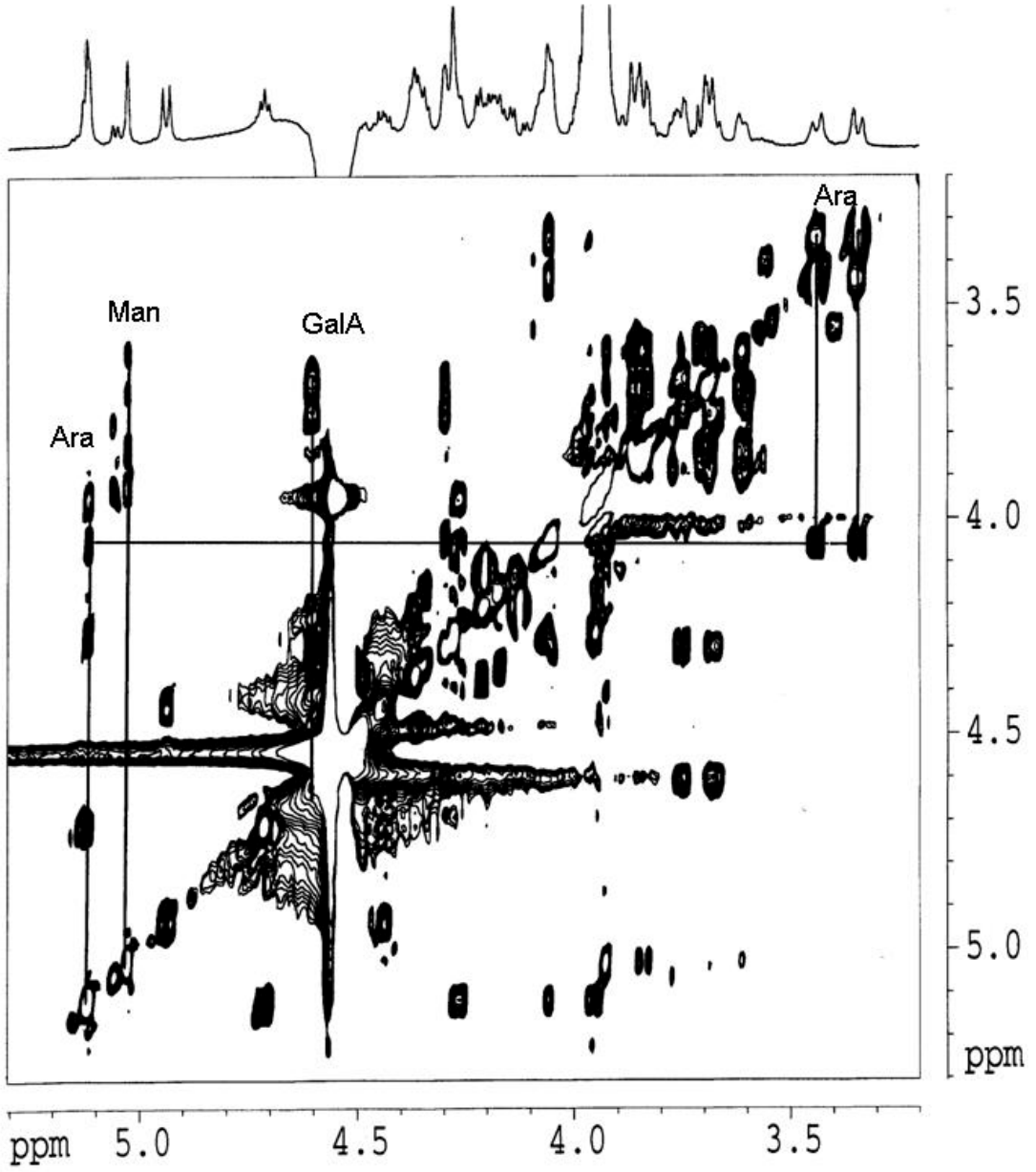


Figure 1S5. Mid-field (H_α and sugar) region of the 2D TOCSY spectrum of 1 in trifluoroethanol-d_2/H_2O (1:1) at 300 K showing assignments of sugar units.

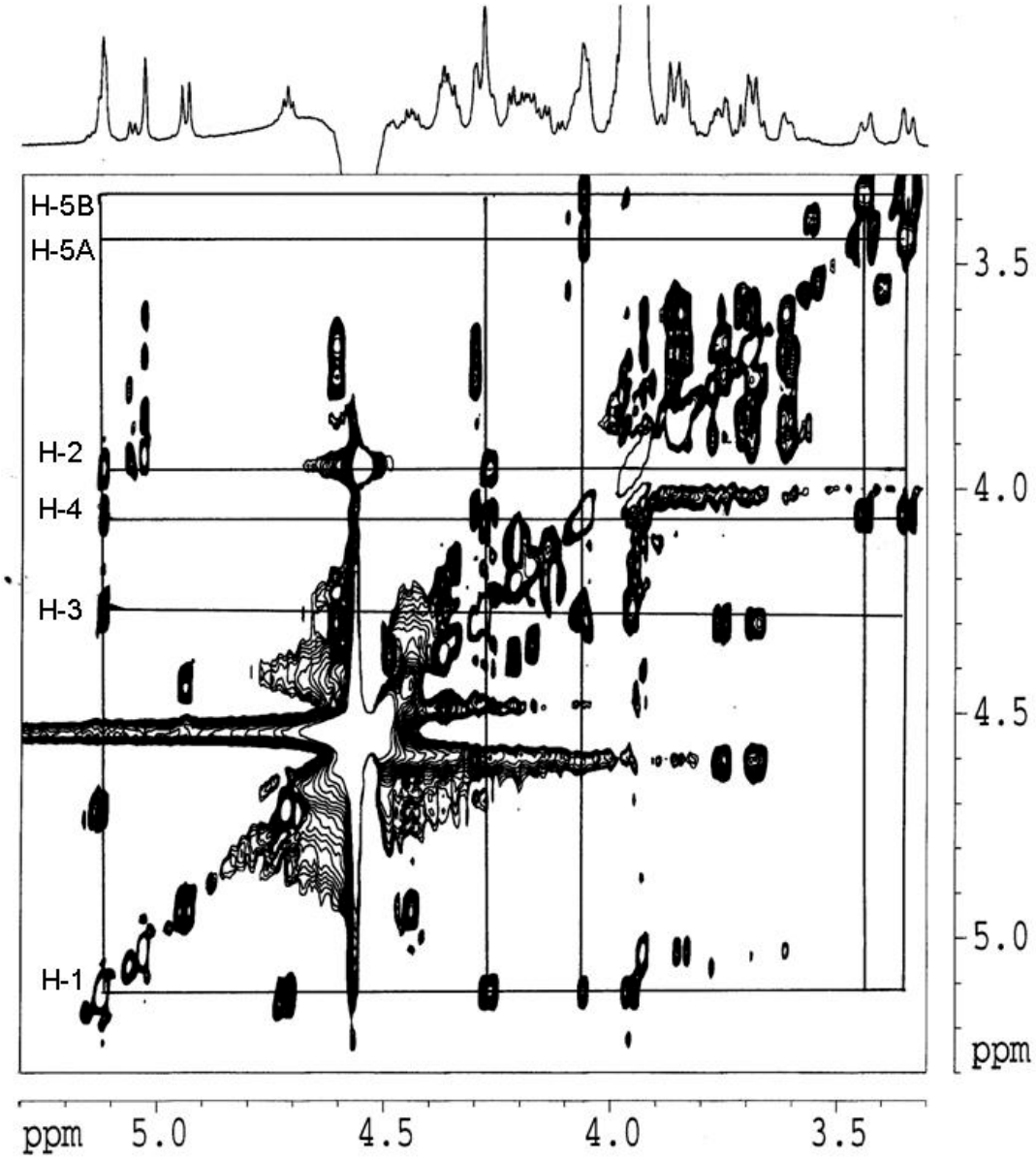


Figure 1S5a. Mid-field (sugar) region of the 2D TOCSY spectrum of 1 in trifluoroethanol-d_2/H_2O (1:1) at 300 K showing assignment of arabinose spin system.

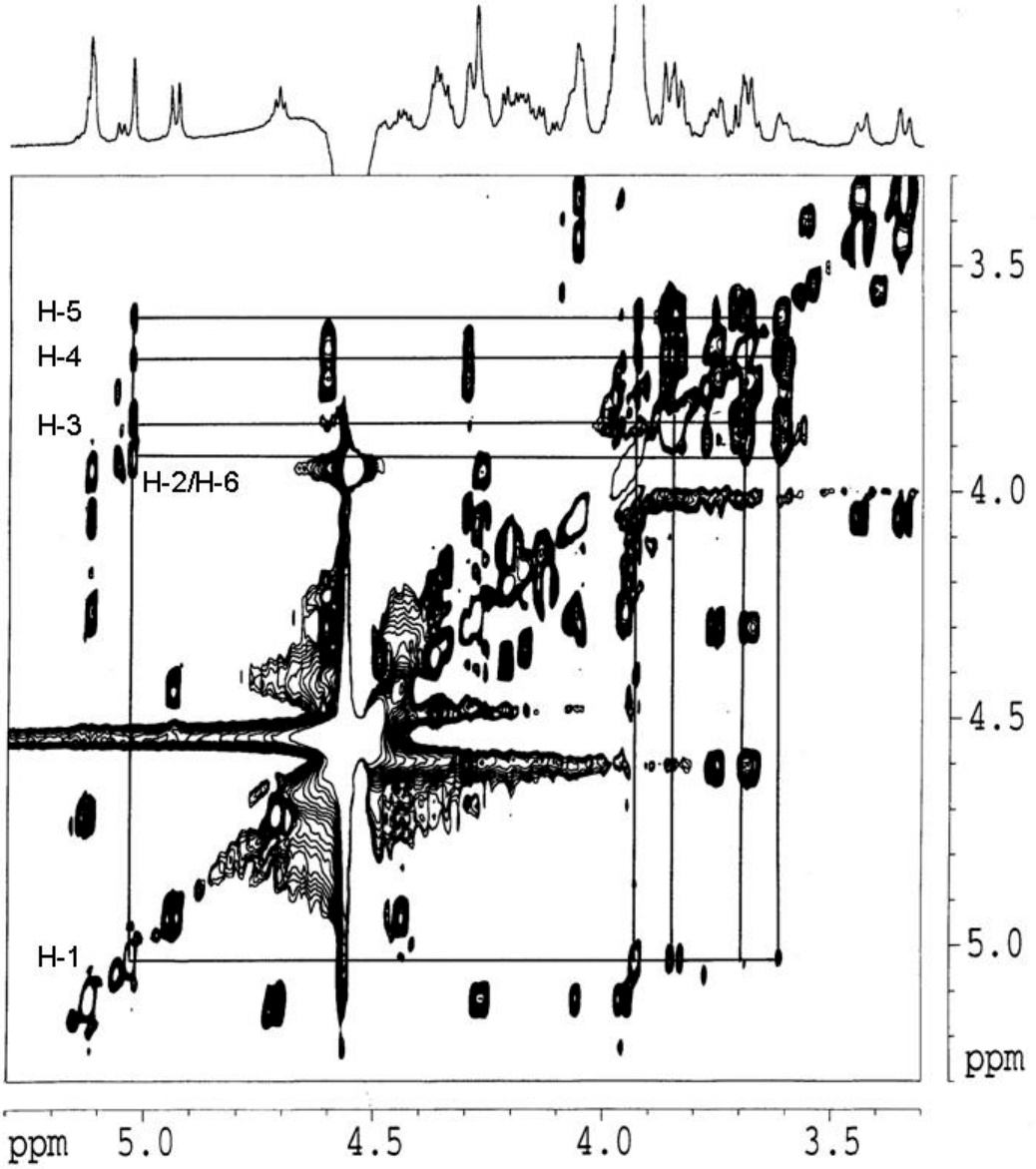


Figure 1S5b. Mid-field (sugar) region of the 2D TOCSY spectrum of 1 in trifluoroethanol-d_2/H_2O (1:1) at 300 K showing assignment of mannose spin system.

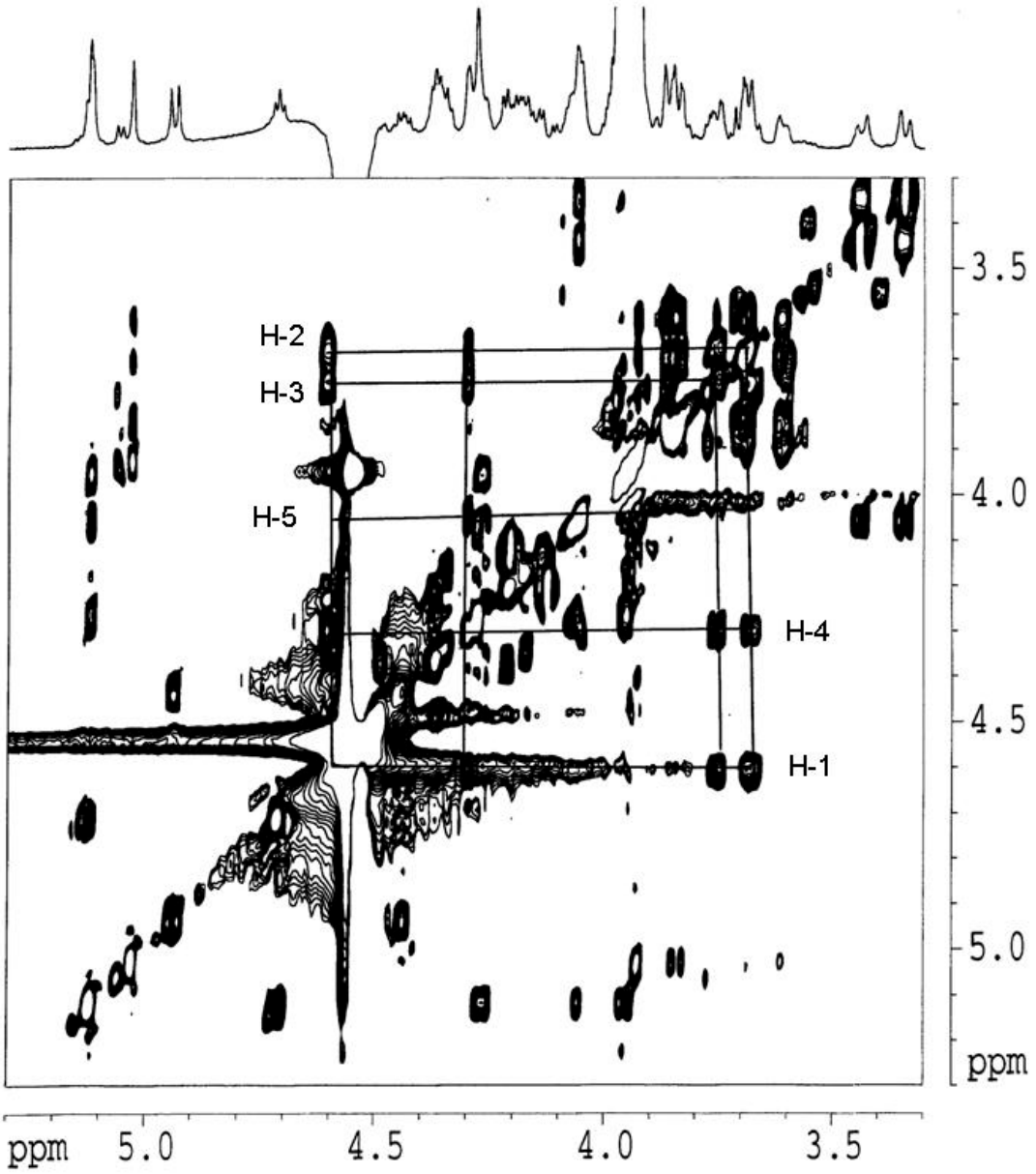


Figure 1S5c. Mid-field (sugar) region of the 2D TOCSY spectrum of 1 in trifluoroethanol-d_2/H_2O (1:1) at 300 K showing assignment of galacturonic acid spin system.

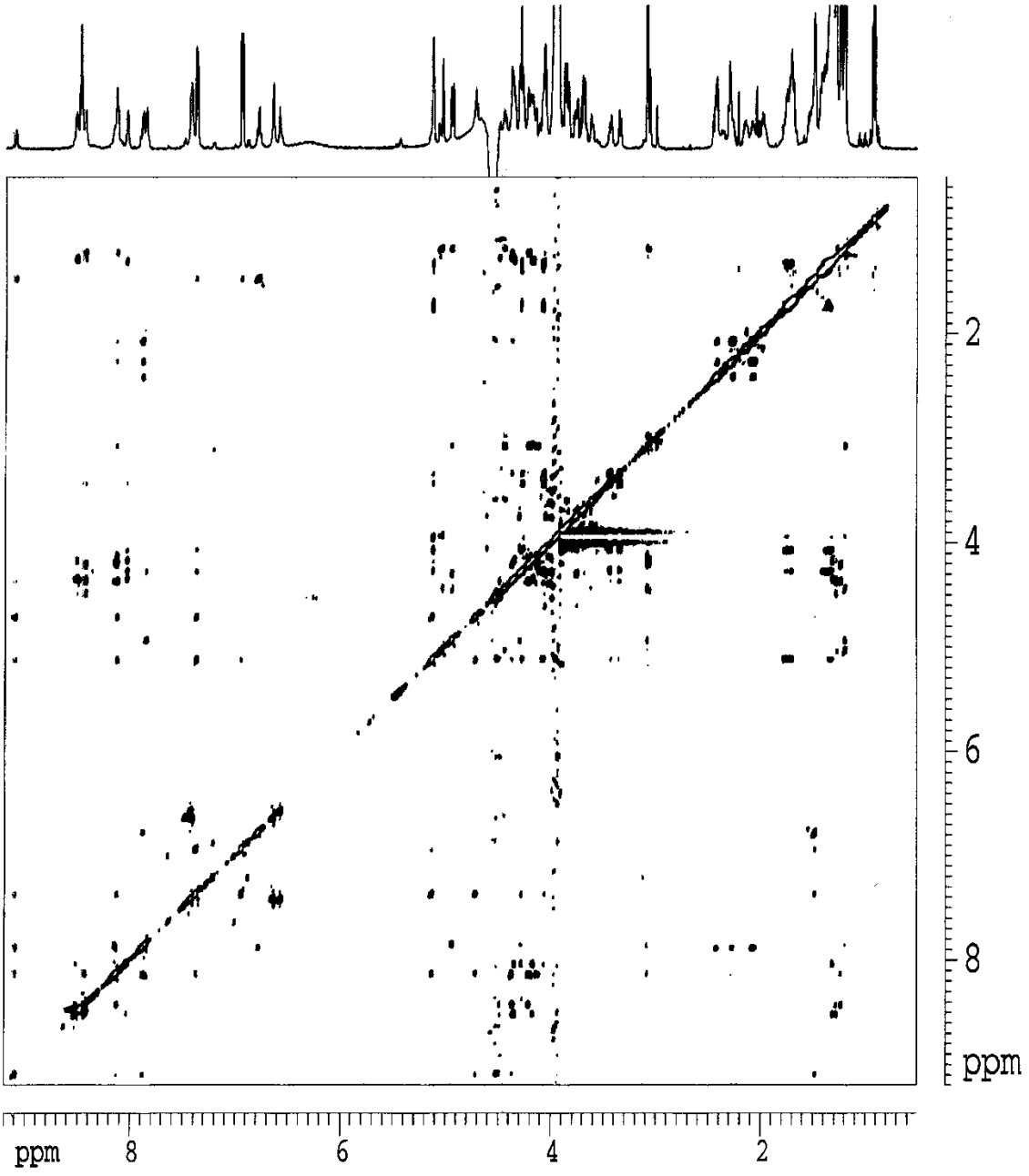


Figure 1S6. 2D NOESY spectrum of 1 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

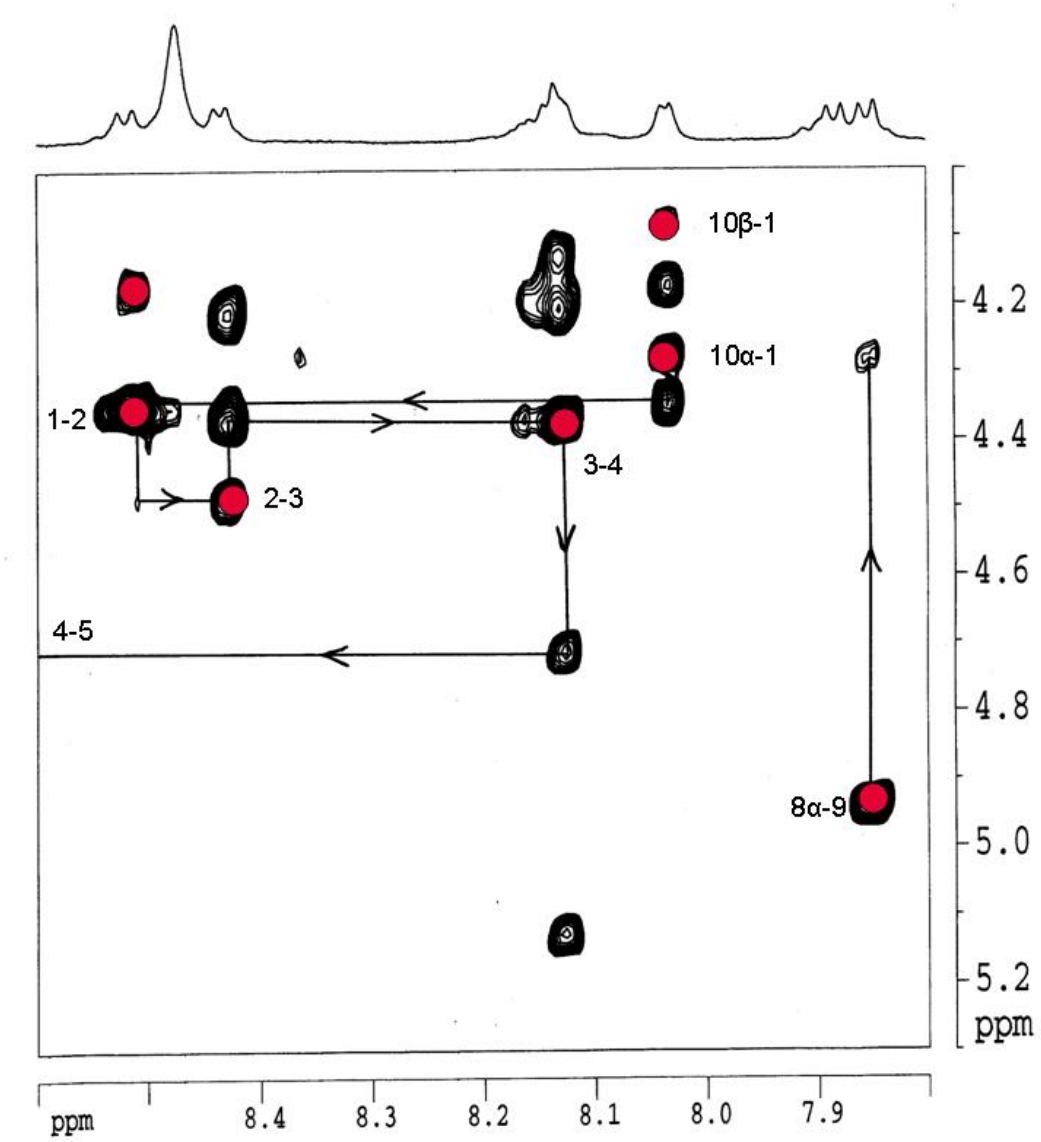


Figure 1S7. Low field 2D NOESY finger-print region of 1 in trifluoroethanol-d_2/H_2O (1:1) at 300 K showing sequence assignment from H_αNH signals (red circles). The sequence signals of 4-5 are outside the region shown. Residue 5 has no H_α and 8 has no NH.

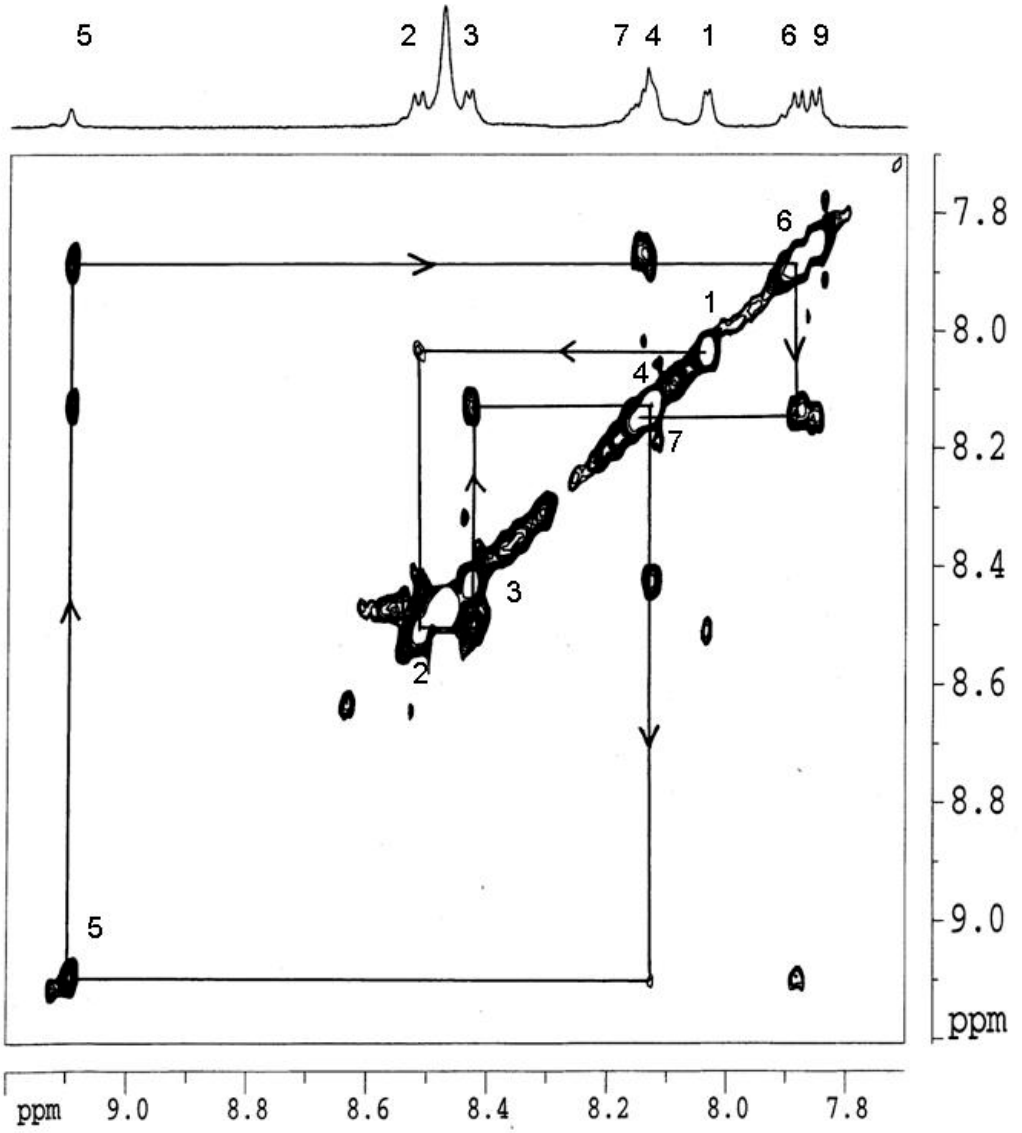


Figure 1S8. NH-NH region of 2D NOESY spectrum of 1 in trifluoroethanol-d_2/H_2O (1:1) at 300 K showing sequence assignment from H_αNH signals. Residue 5 has no H_α and 8 has no NH.

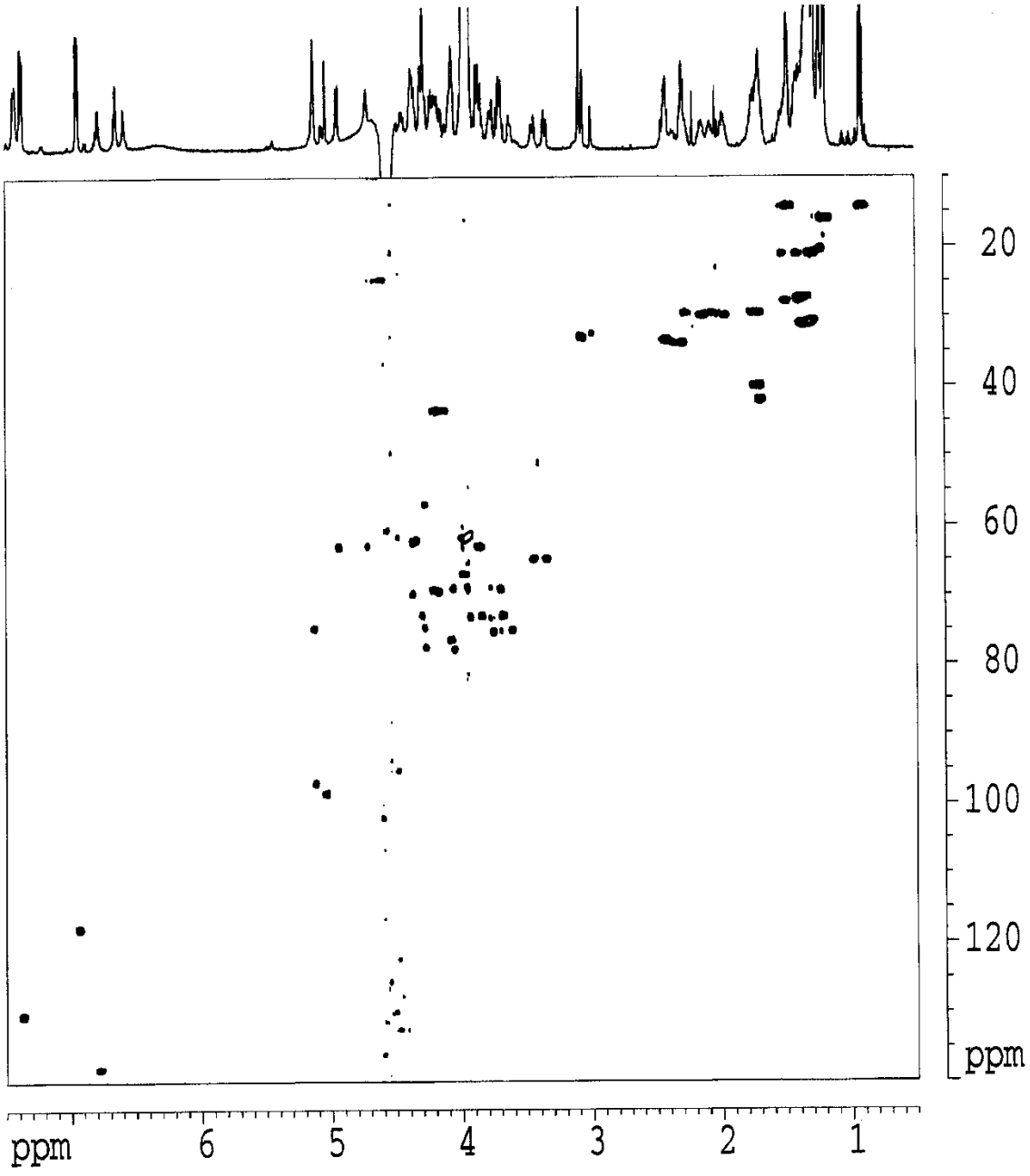


Figure 1S9. 2D HSQC spectrum of 1 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

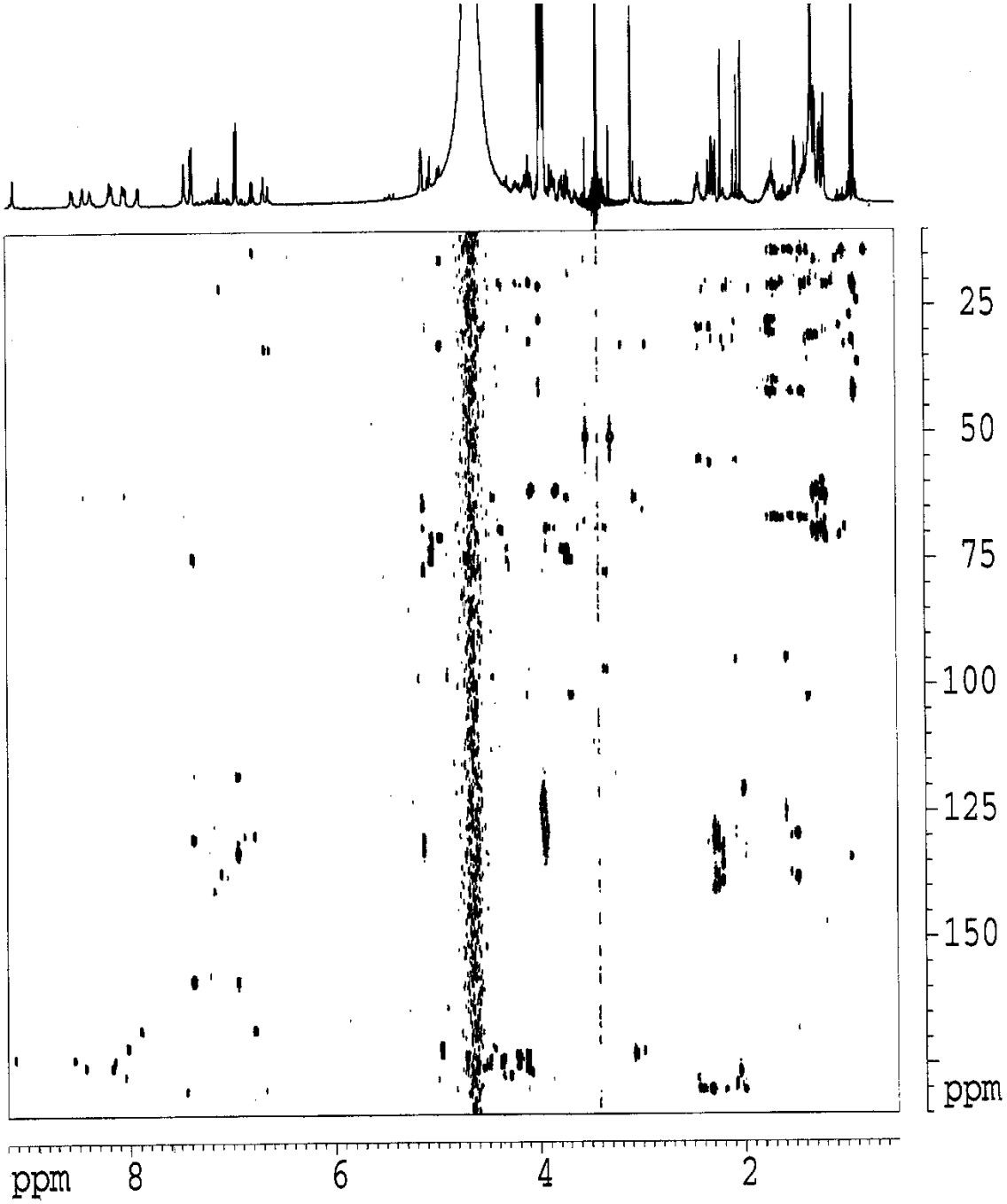


Figure 1S10. 2D HMBC spectrum of 1 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

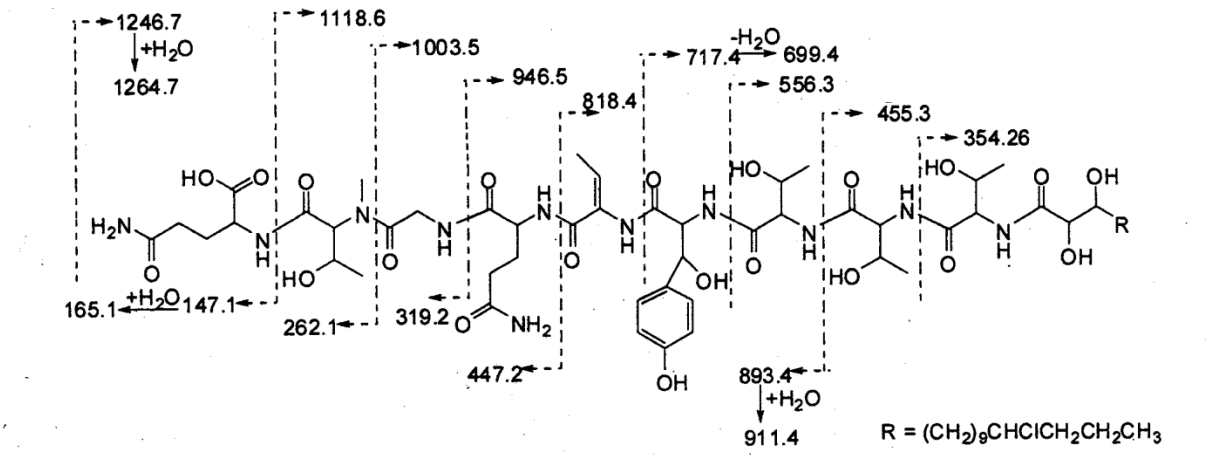


Figure 1S11. MS^n fragmentation ions from the ESI-MS of 1 that confirm the peptide sequence.

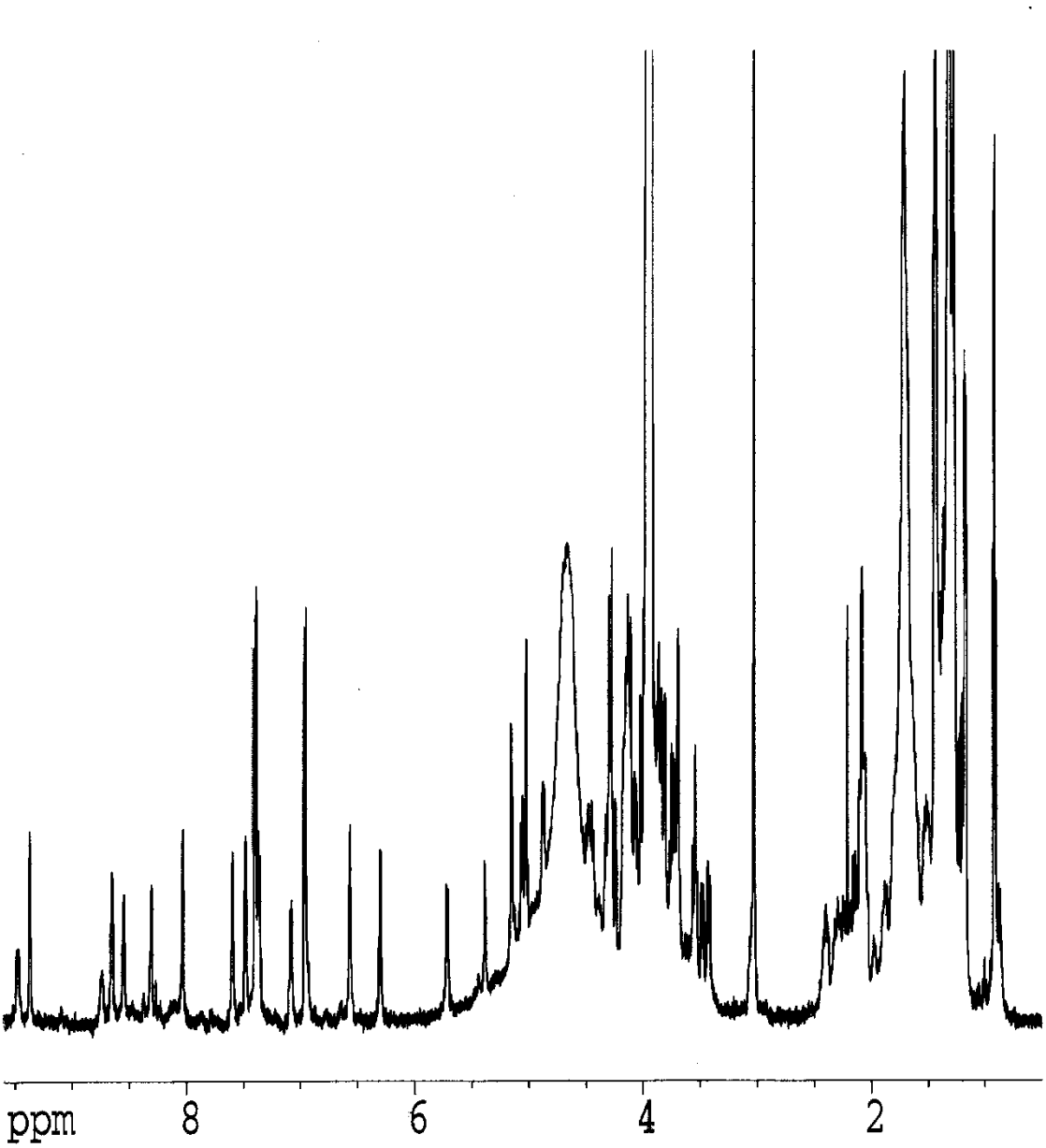


Figure 2S1. ^{1}H NMR spectrum of 2 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

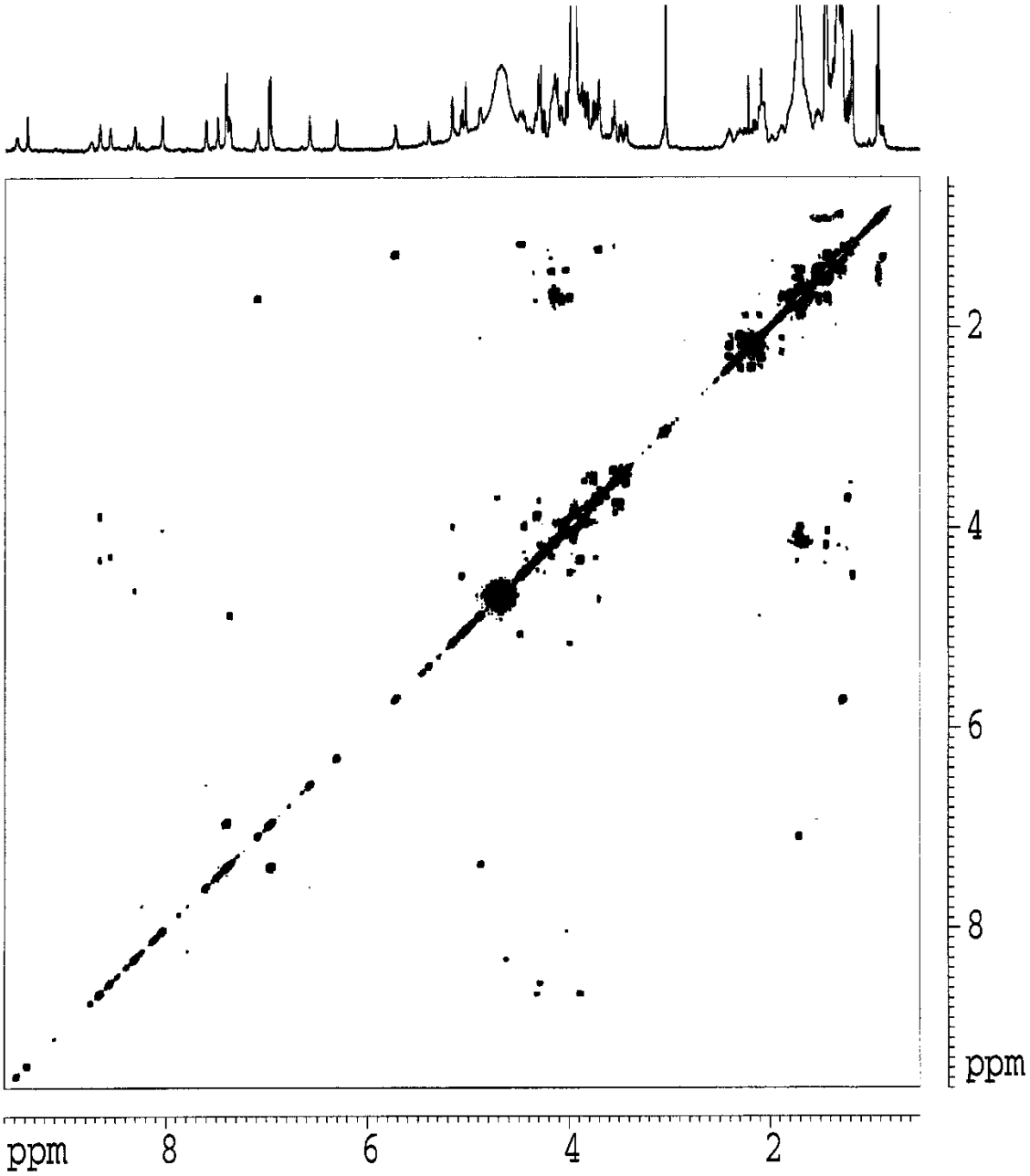


Figure 2S2. 2D COSY spectrum of 2 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

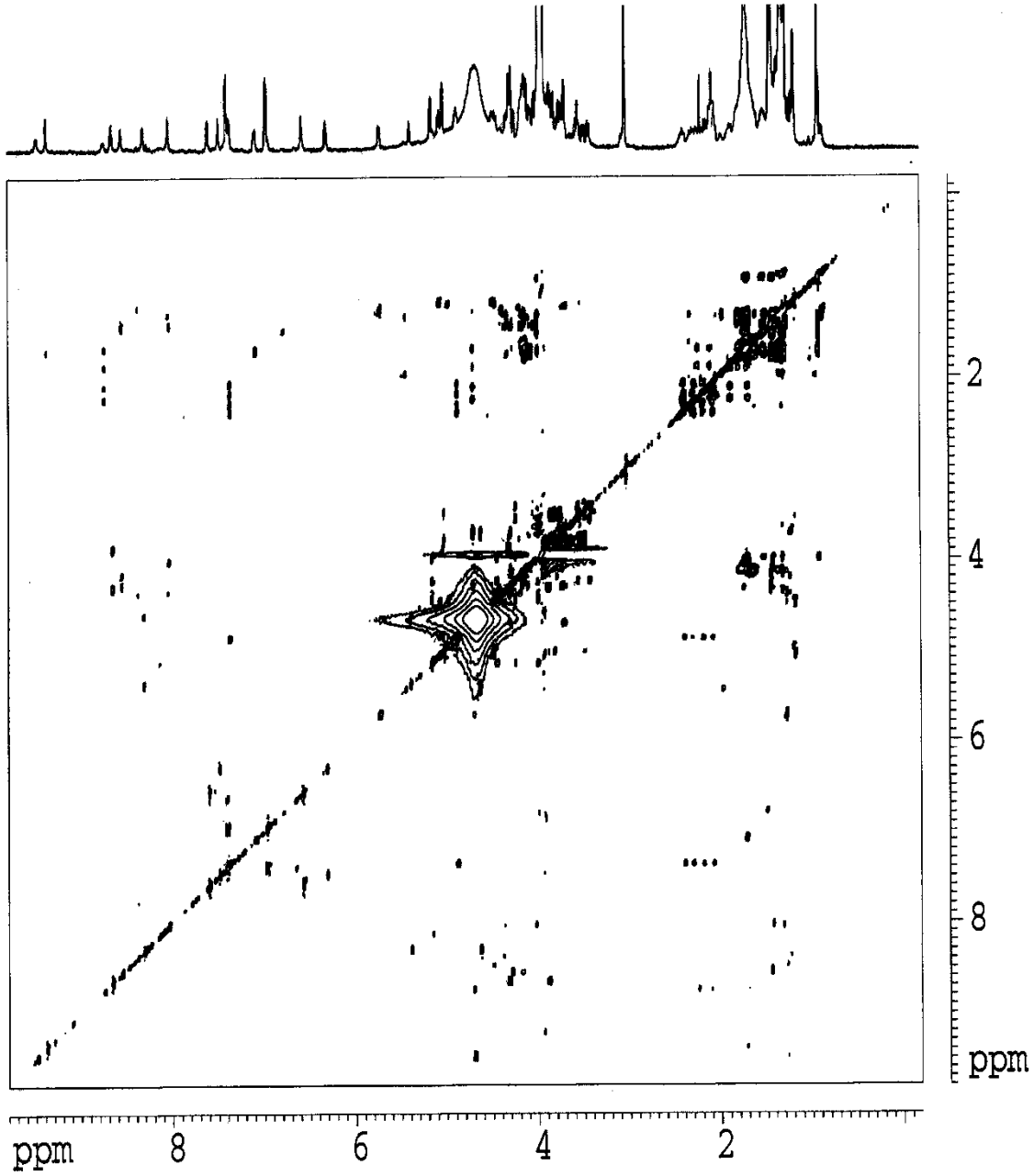


Figure 2S3. 2D TOCSY spectrum of 2 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

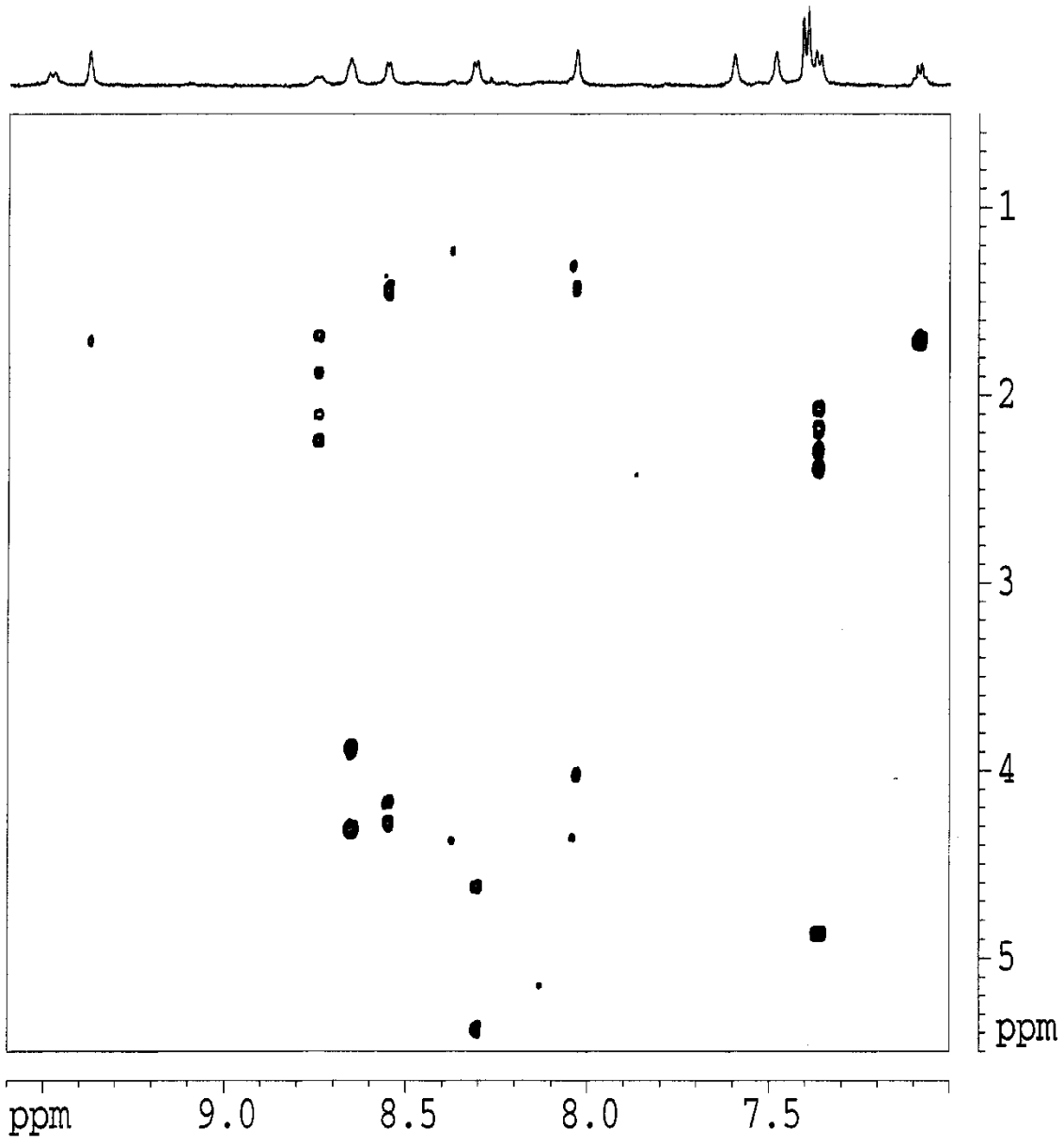


Figure 2S4. Low-field region of the 2D TOCSY spectrum of 2 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

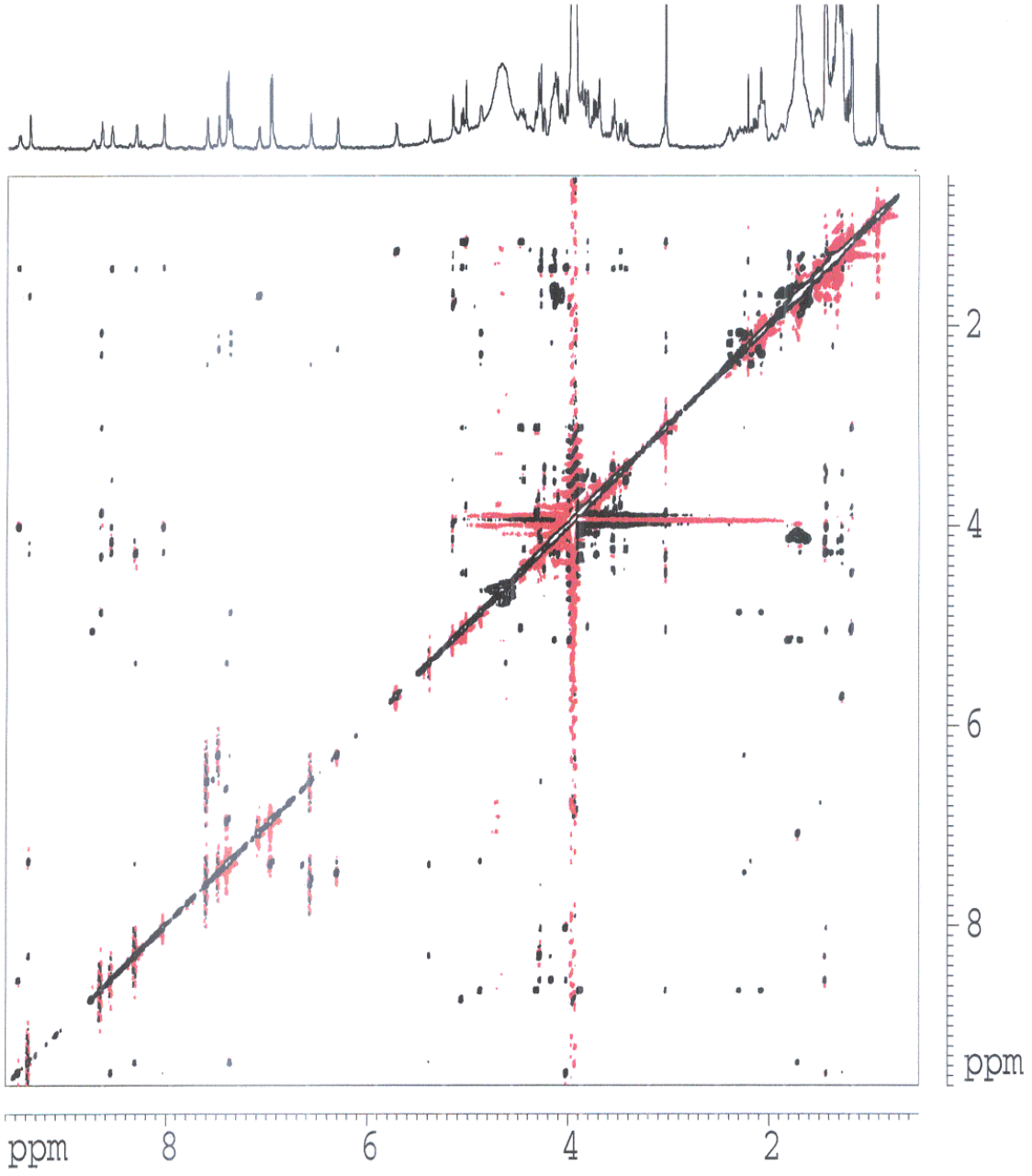


Figure 2S5. 2D ROESY spectrum of 2 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

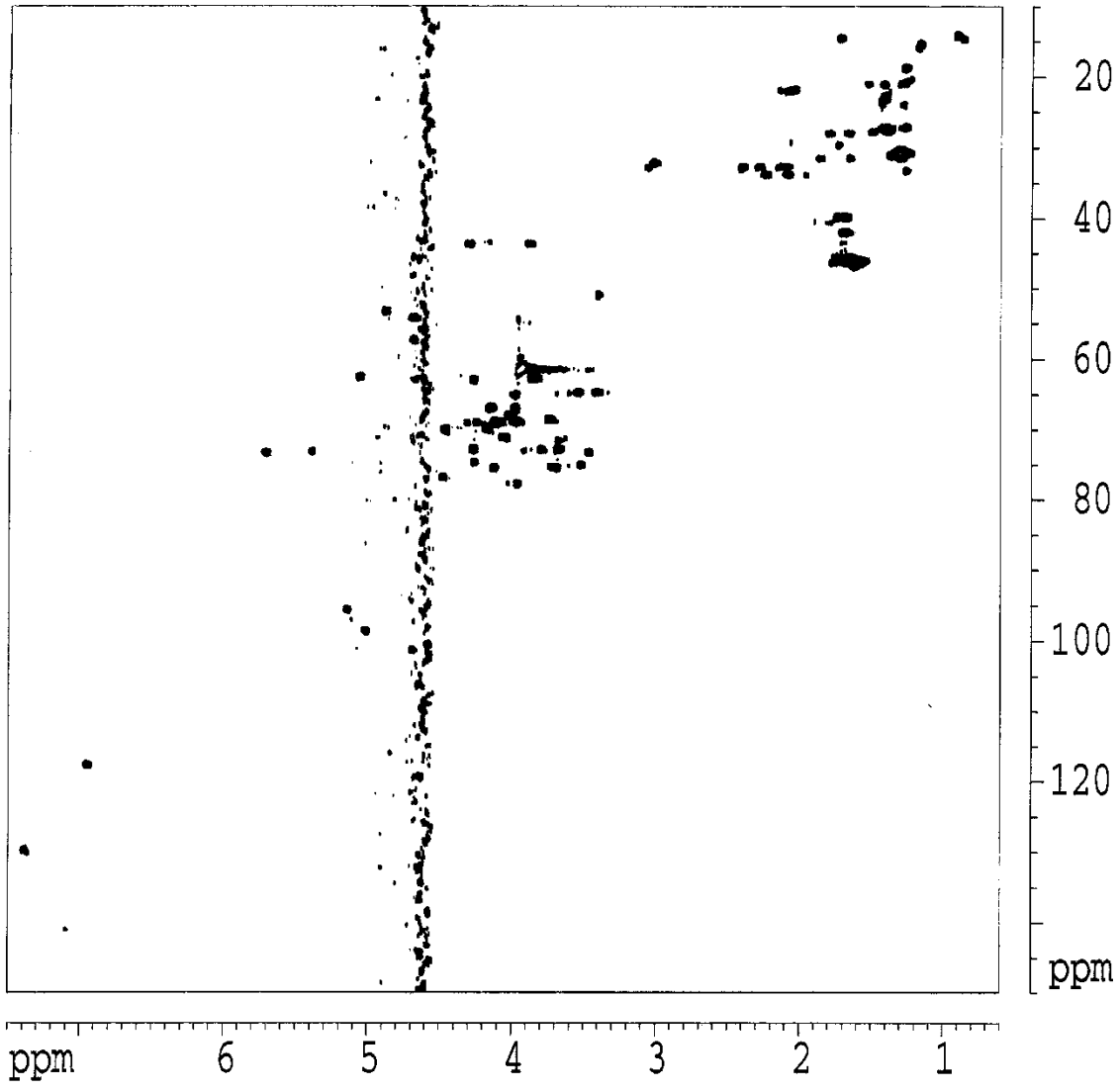


Figure 2S6. 2D HSQC spectrum of 2 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

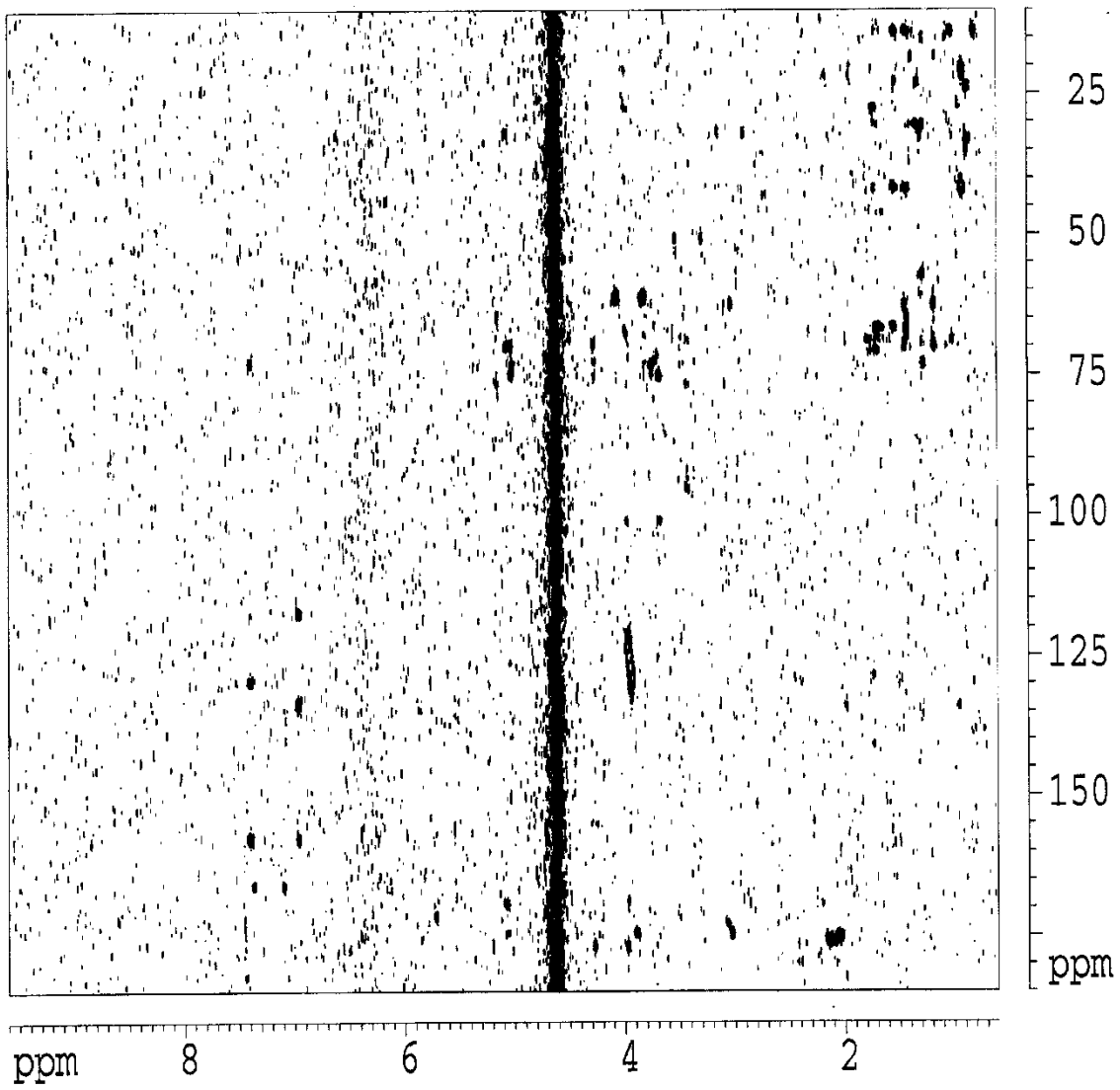


Figure 2S7. 2D HMBC spectrum of 2 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

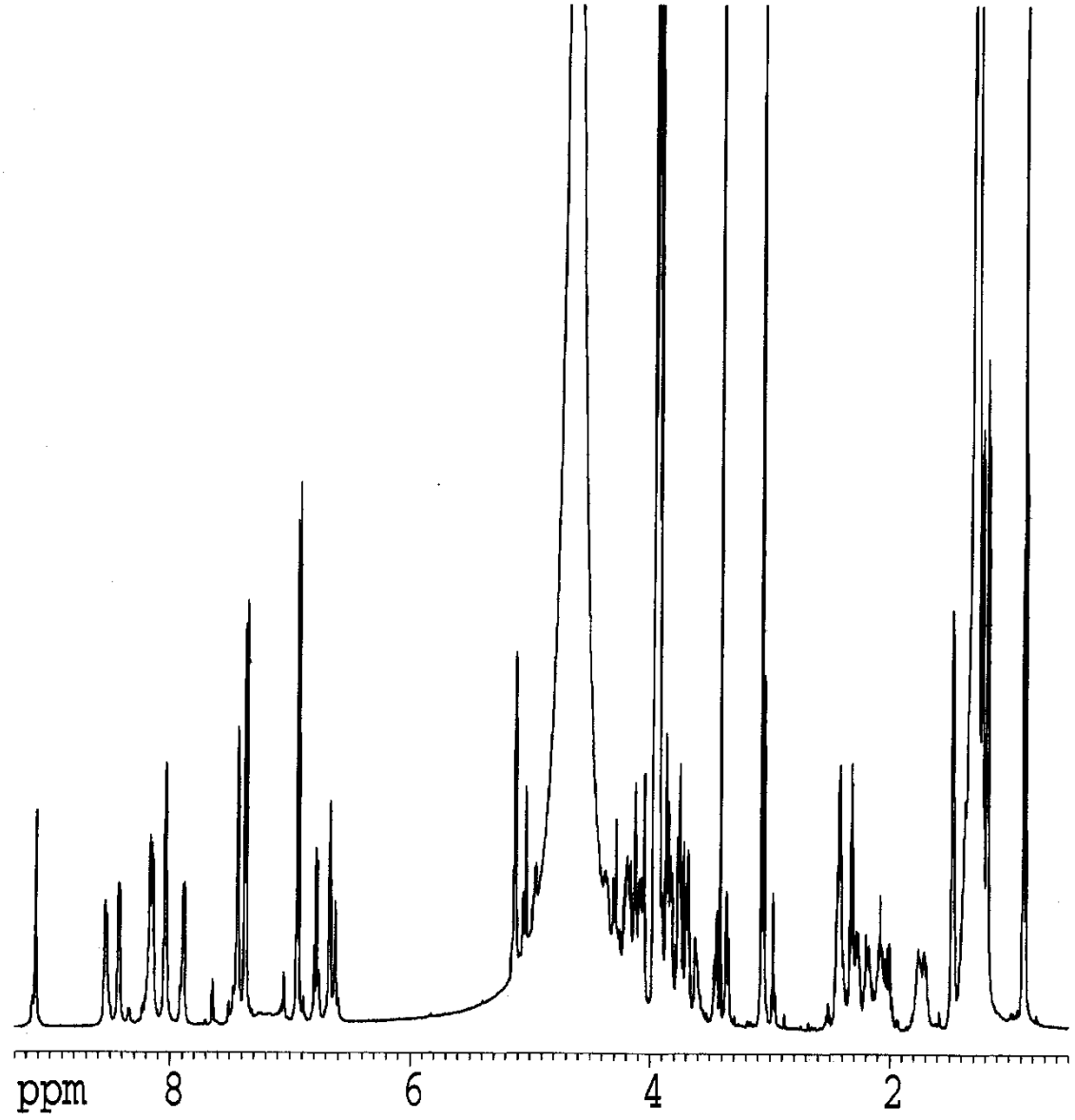


Figure 3S1. 1D ^{1}H NMR spectrum of 3 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

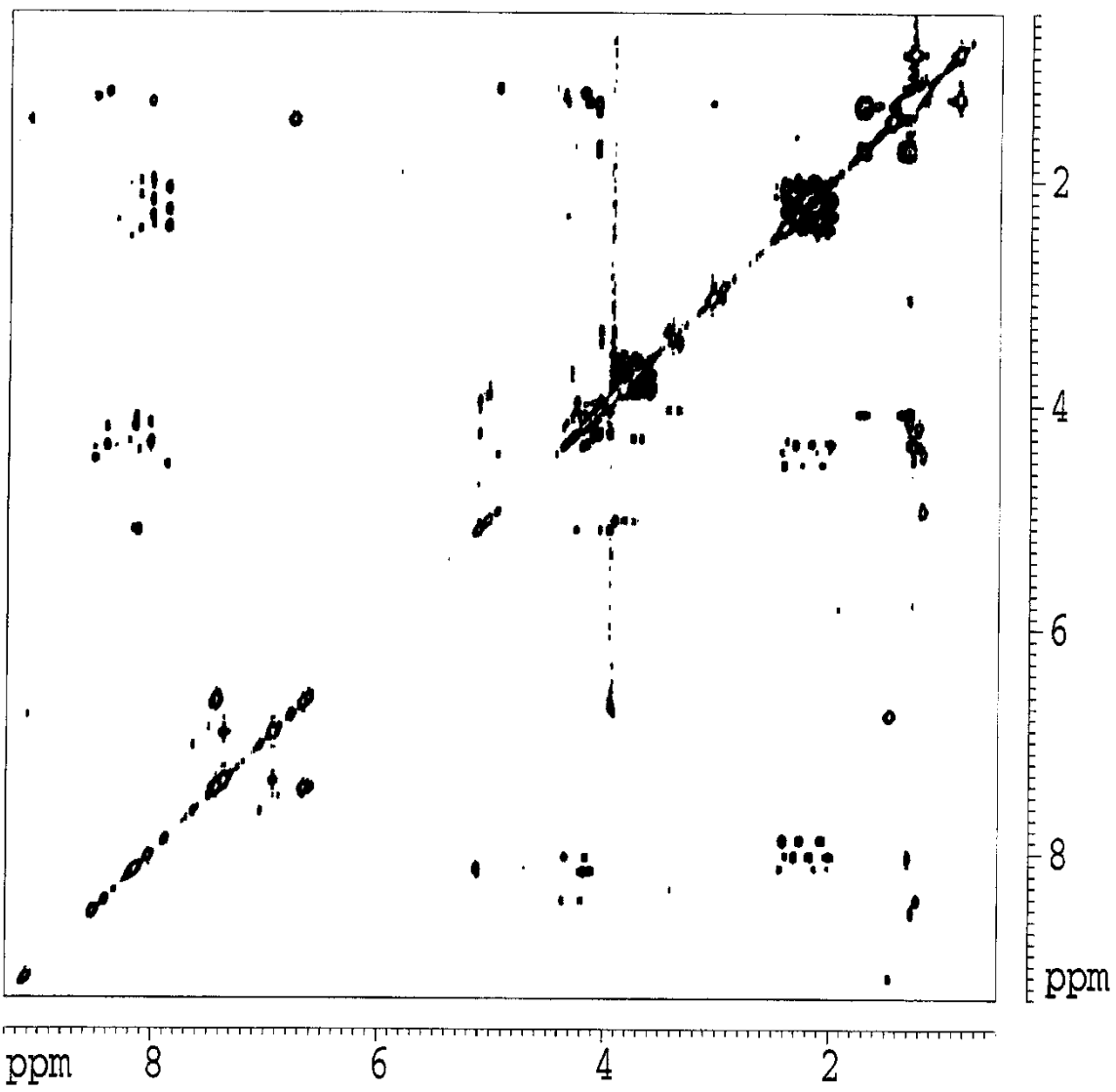


Figure 3S2. 2D TOCSY spectrum of 3 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

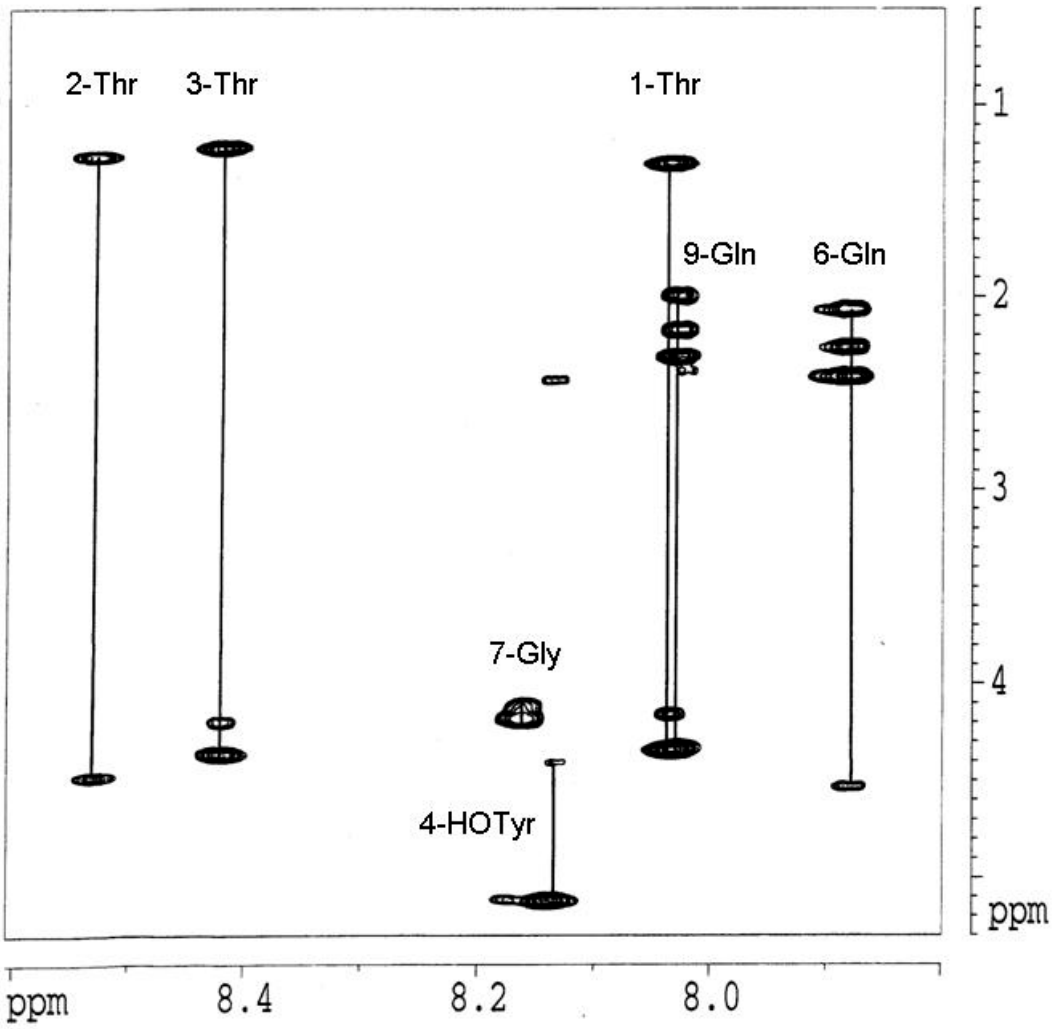


Figure 3S3. Low-field region of the 2D TOCSY spectrum of 3 in trifluoroethanol-d_2/H_2O (1:1) at 300 K showing amino acid spin systems.

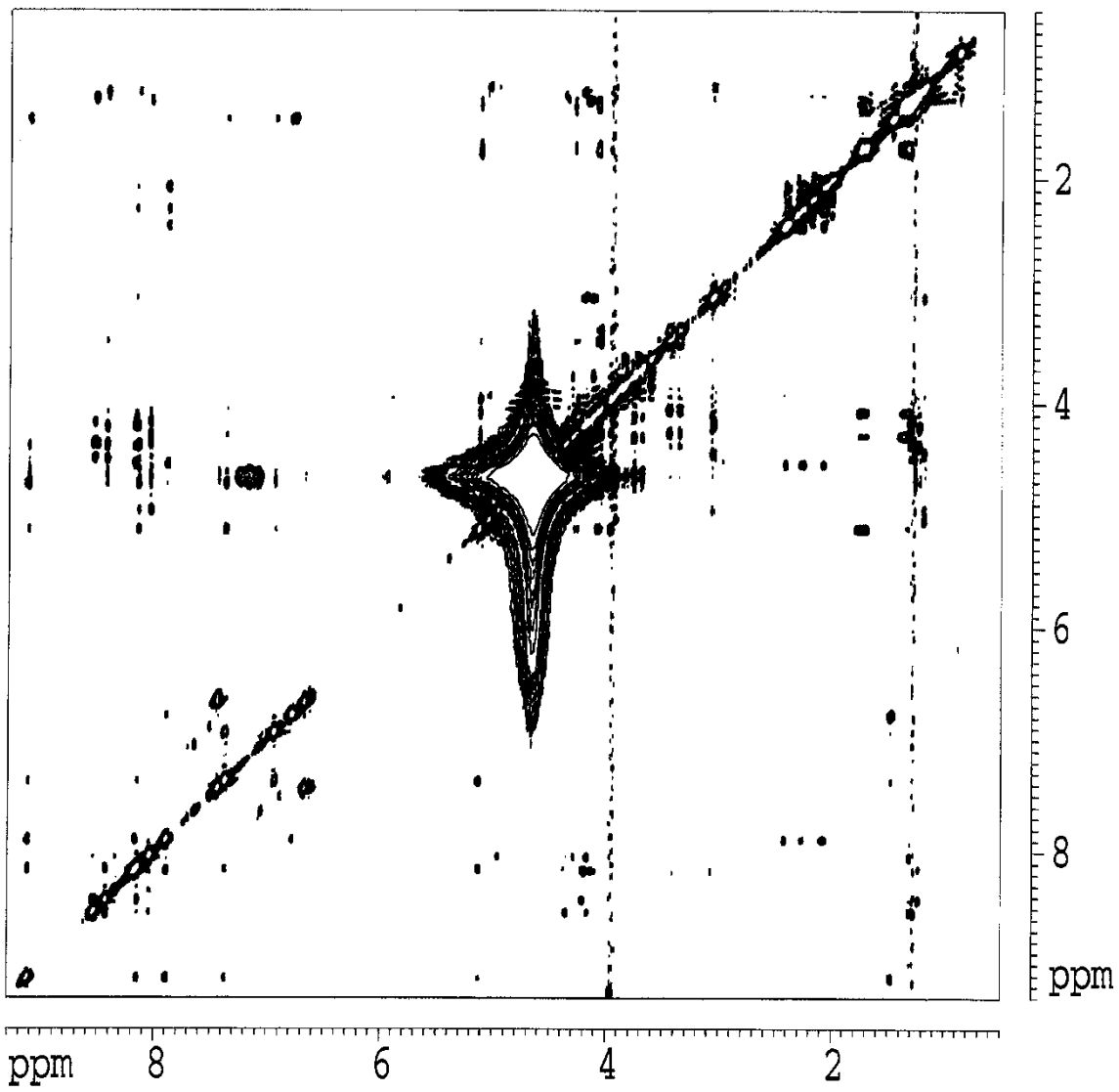


Figure 3S4. 2D NOESY spectrum of 3 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

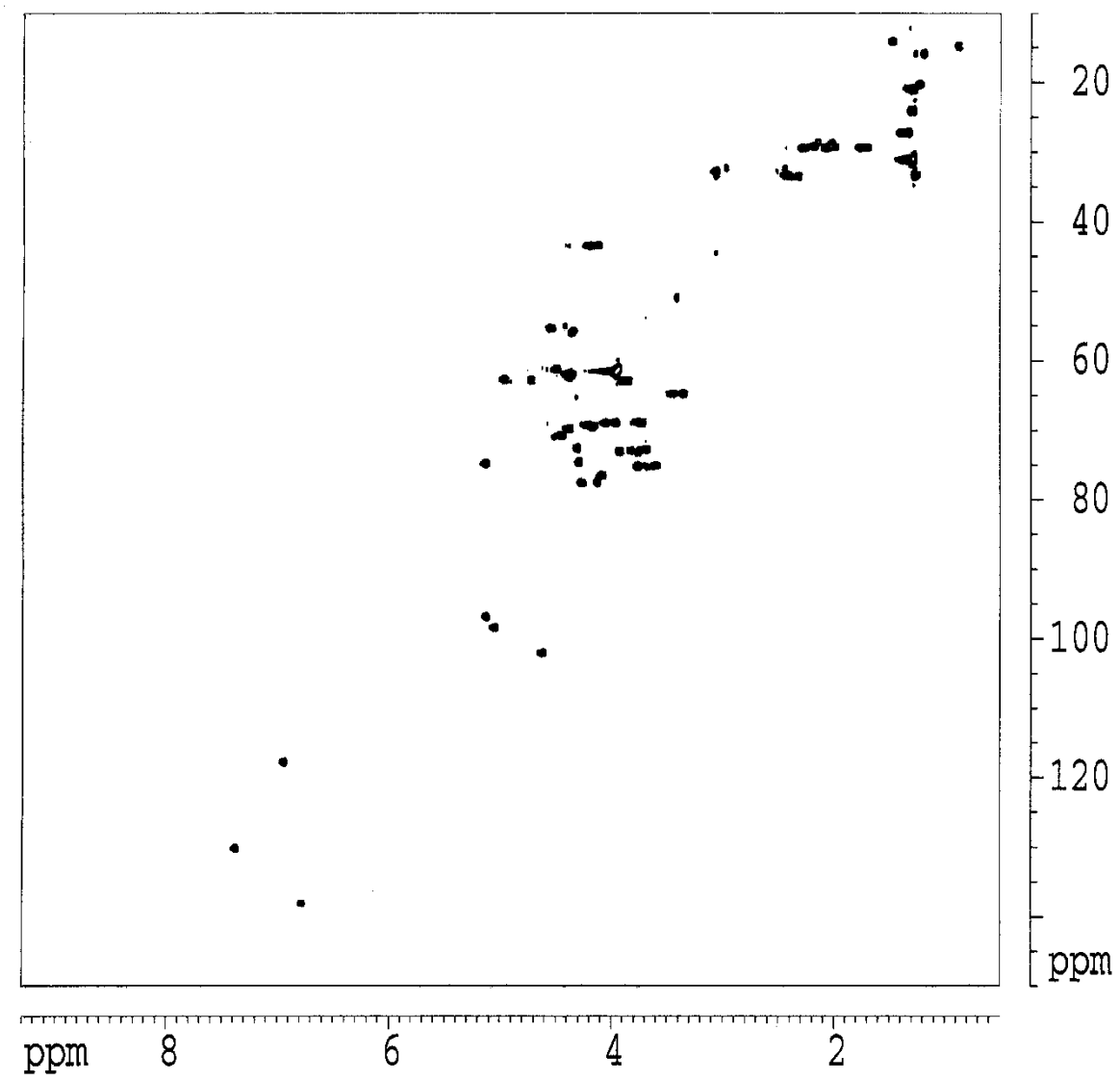


Figure 3S5. 2D HSQC spectrum of 3 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

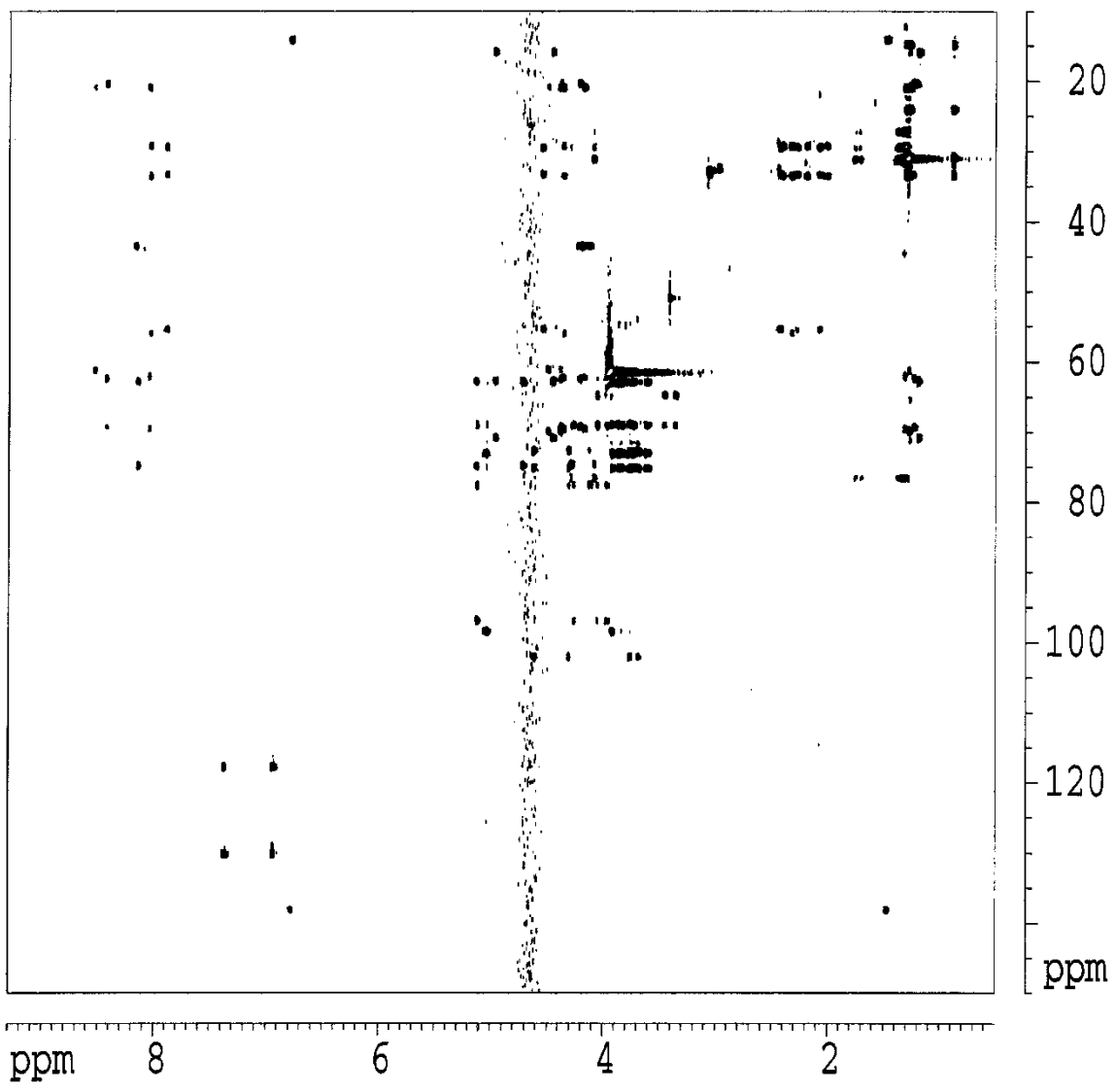


Figure 3S6. 2D HSQC-TOCSY spectrum of 3 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

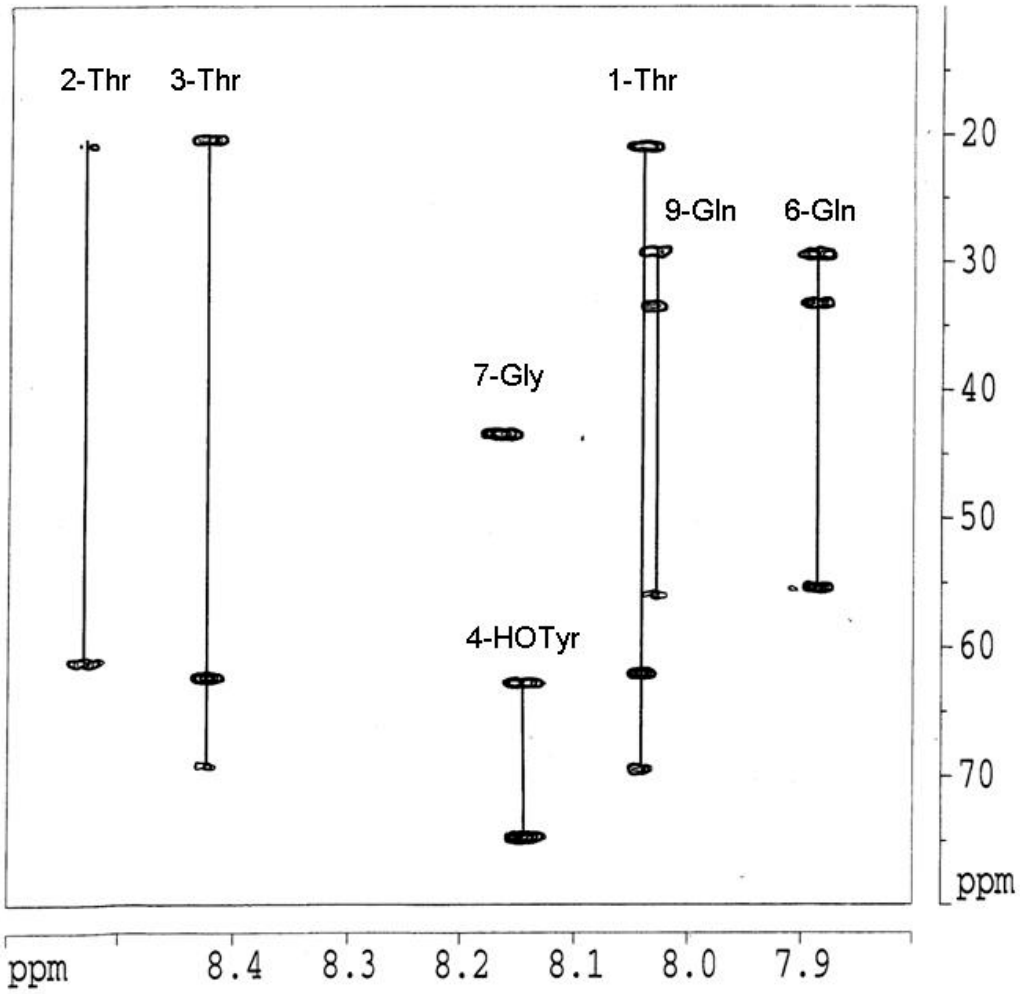


Figure 3S7. Low-field 1H region of 2D HSQC-TOCSY spectrum of **3** in trifluoroethanol-d_2/H_2O (1:1) at 300 K showing assignment of amino acid spin systems.

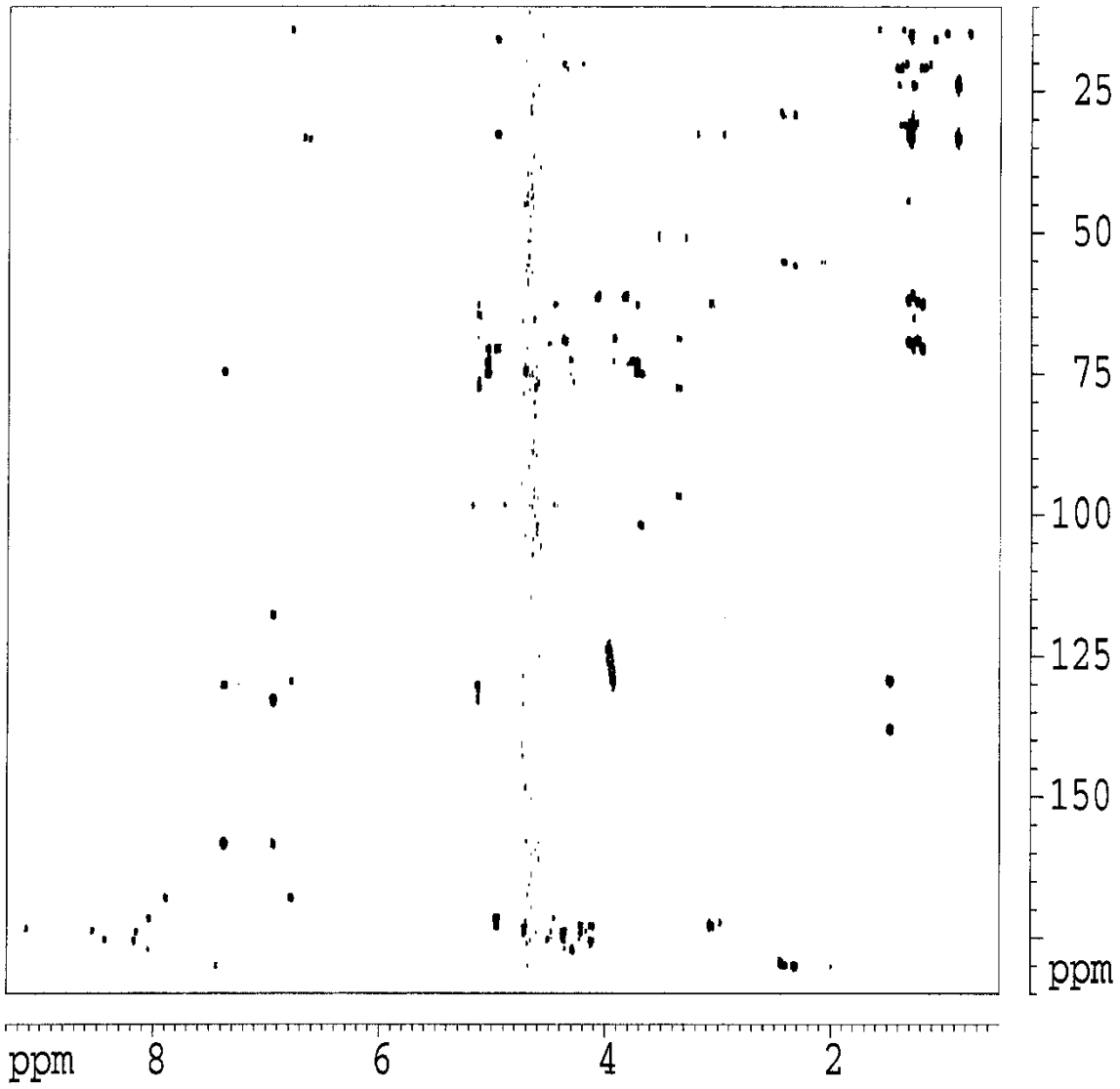


Figure 3S8. 2D HMBC spectrum of 3 in trifluoroethanol-d_2/H_2O (1:1) at 300 K.

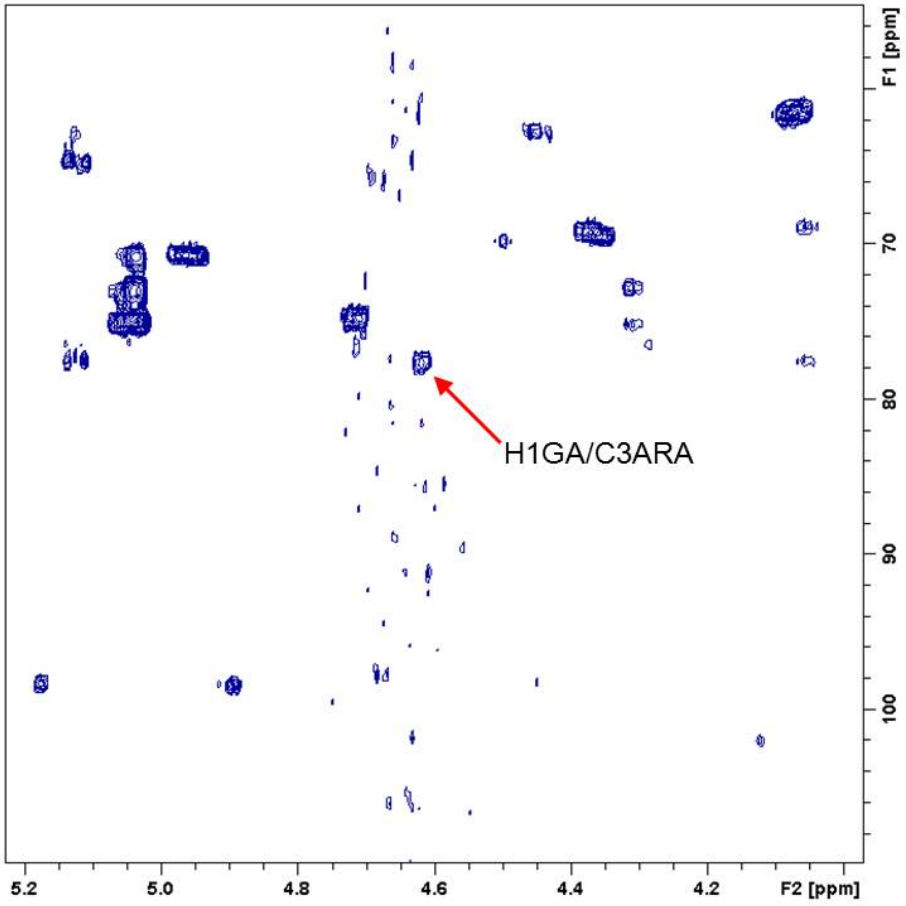


Figure 3S9. Expansion of the sugar region of the 2D HMBC spectrum of 3 showing the correlation of the anomeric proton of the galacturonic acid moiety with C3 of the arabinose moiety.

V. IR spectra of pure compounds from Bio 33

The IR spectra were recorded using a NICOLET IR200 Fourier transform infrared spectrometer (FT-IR) (Thermo Electron Corporation, USA).

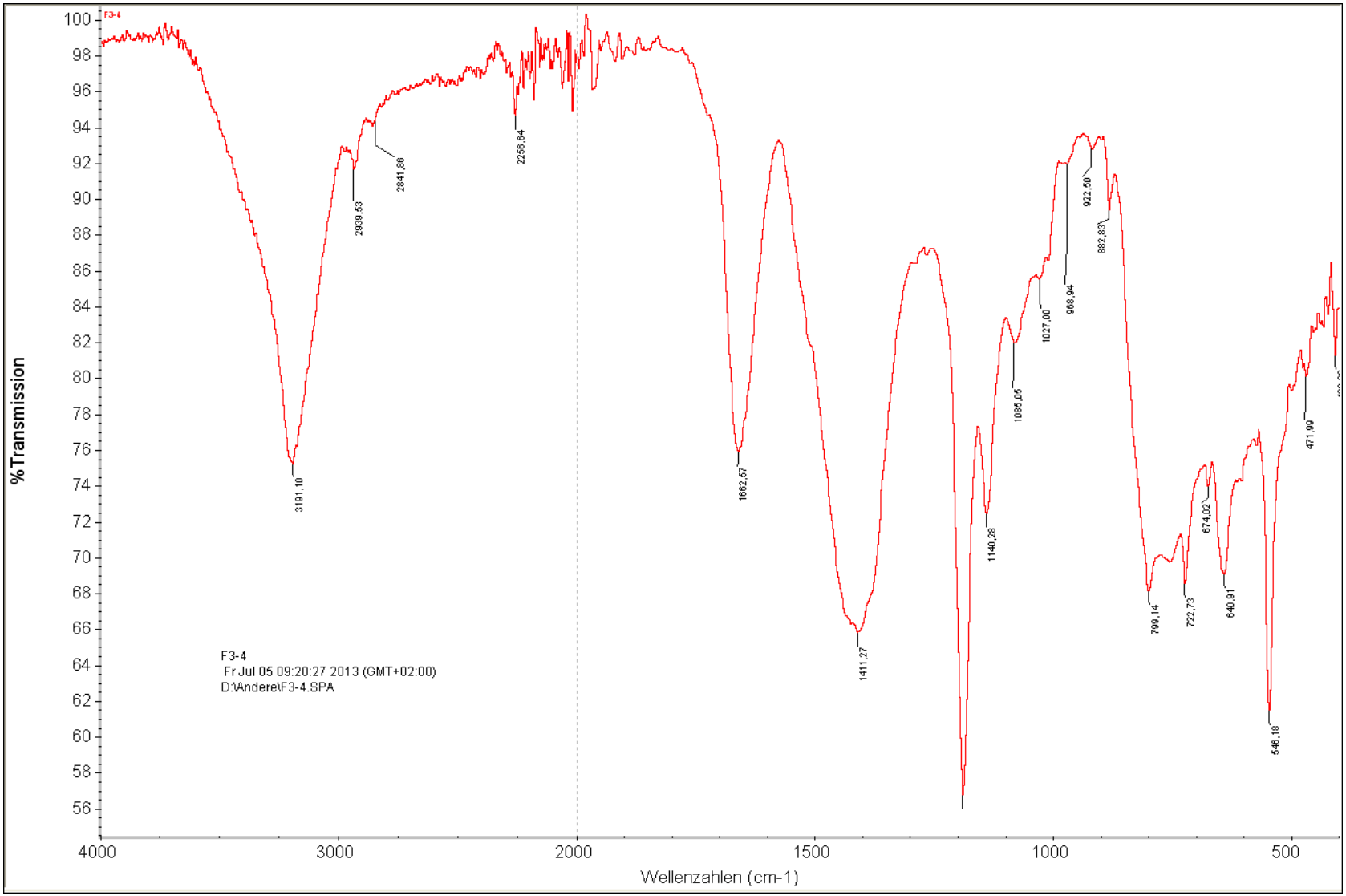


Figure 4S1. Infrared spectrum of compound 1.

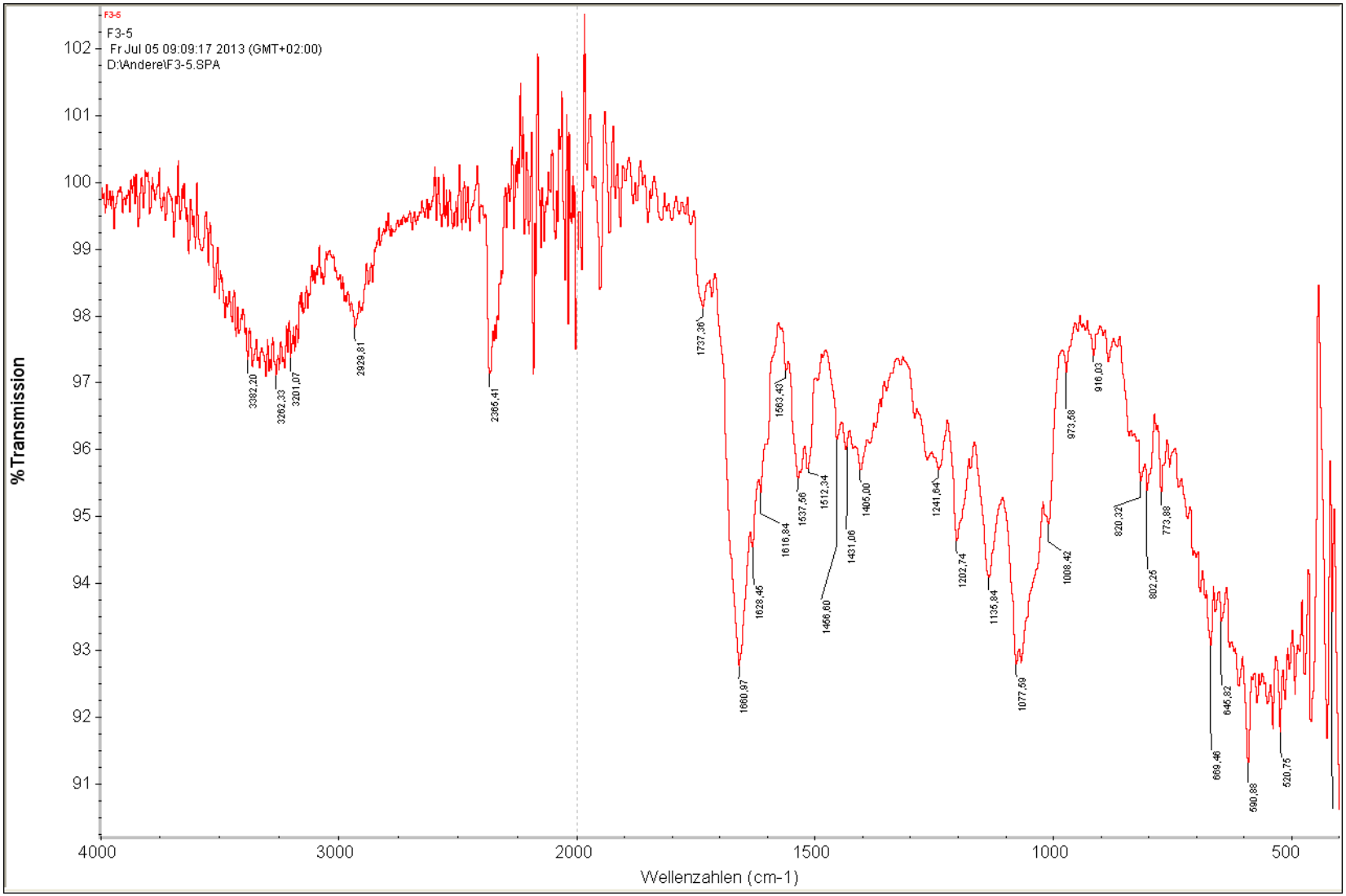


Figure 4S2. Infrared spectrum of compound 2.

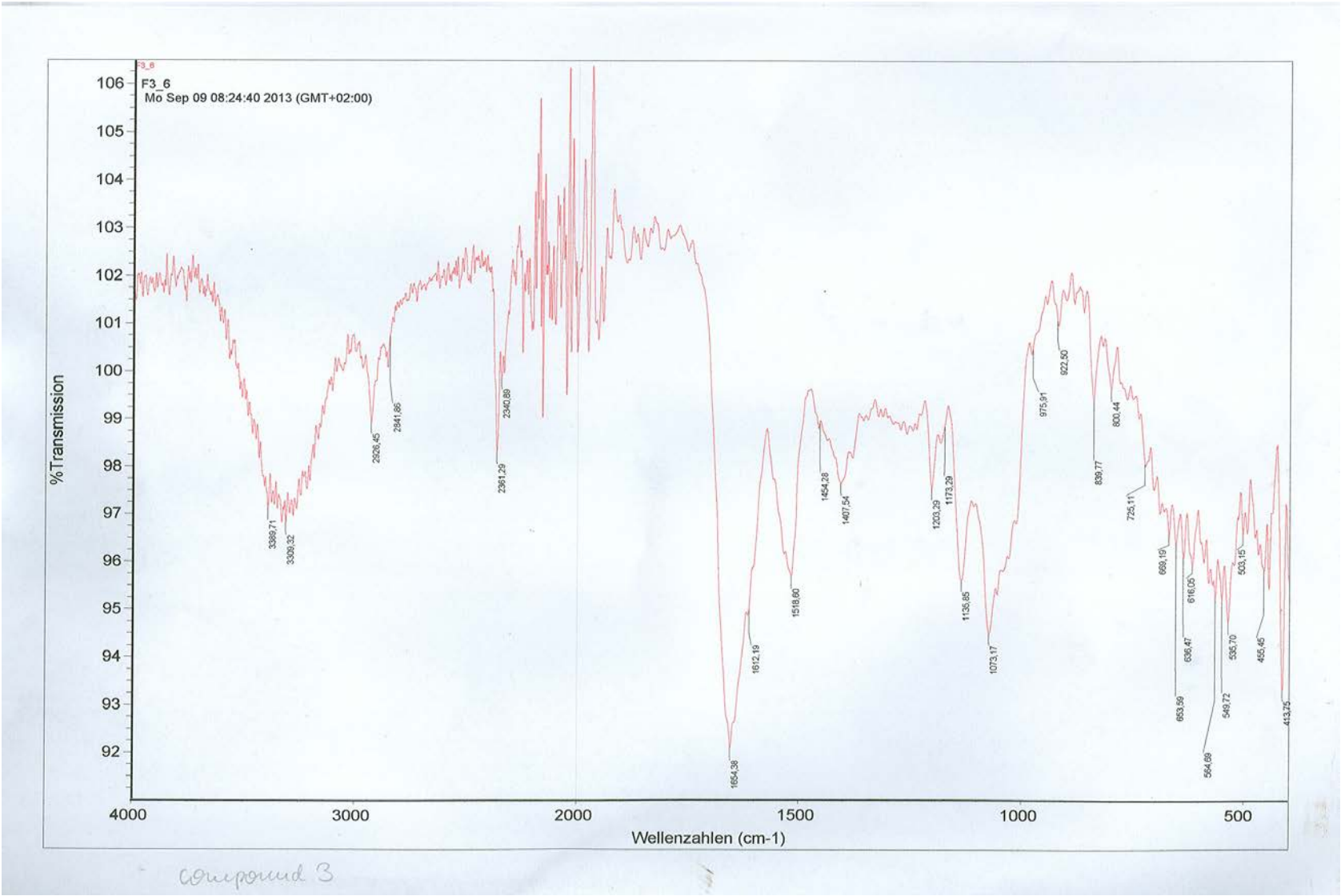


Figure 4S3. Infrared spectrum of compound 3.

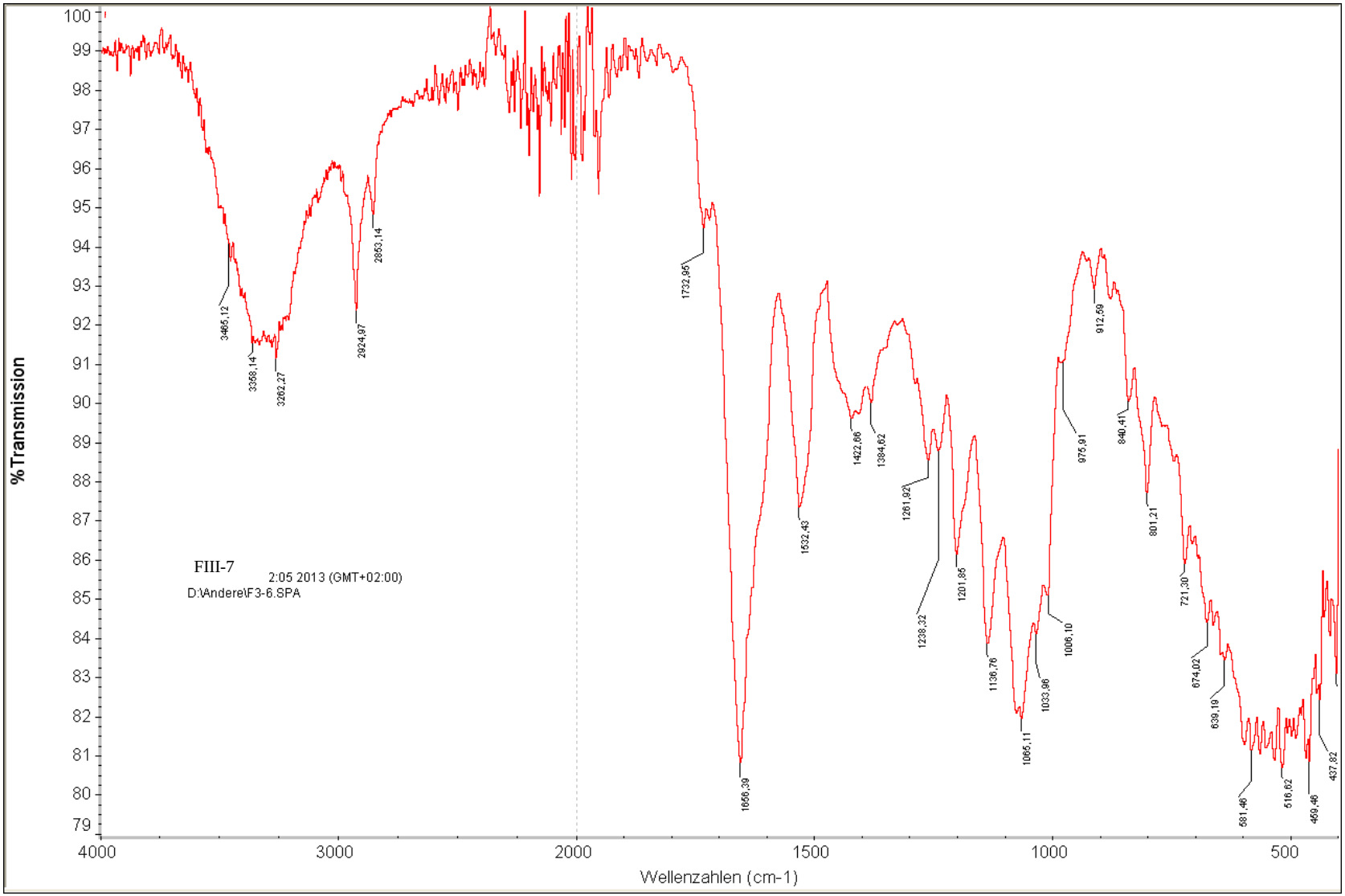


Figure 4S4. Infrared spectrum of compound 4.

VI. HPLC-chromatograms of the isolated compounds from strain TVN40

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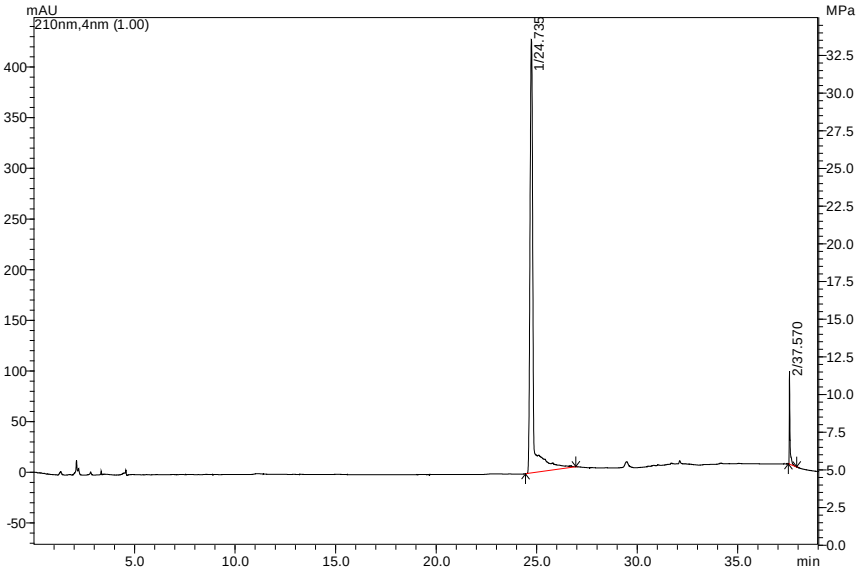
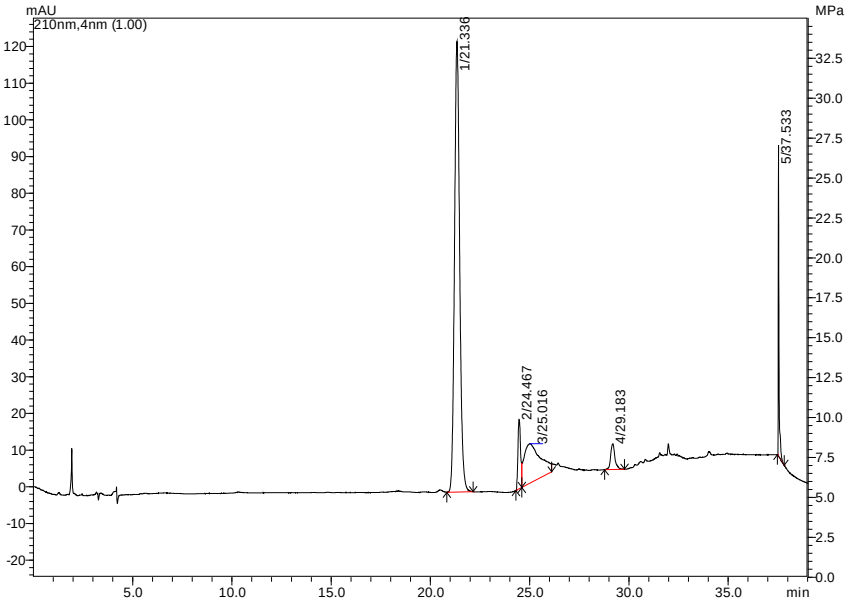
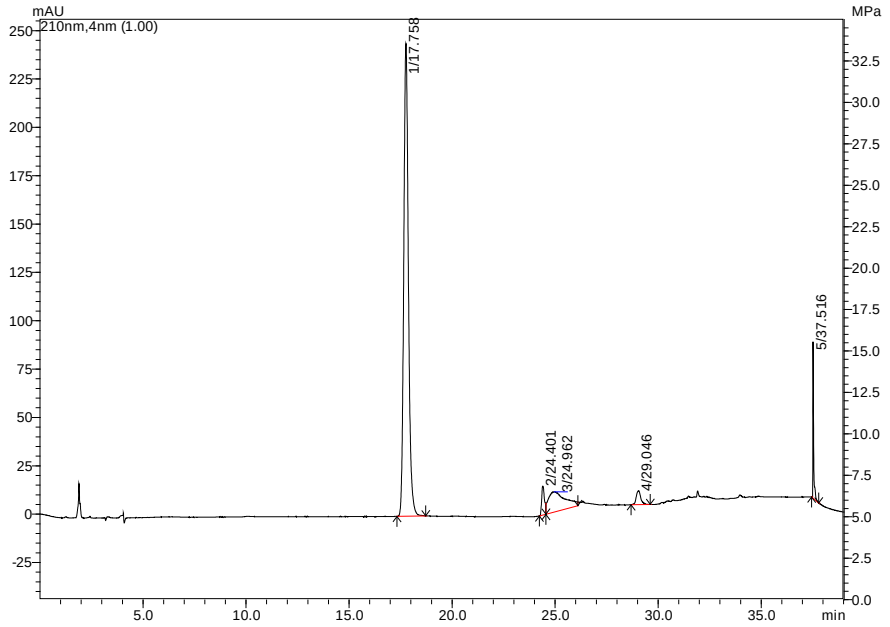


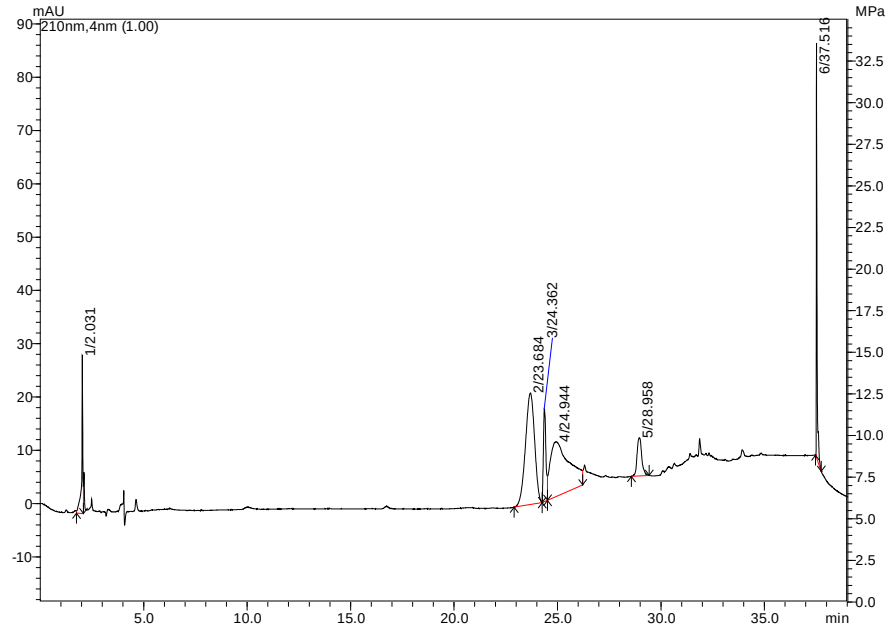
Compound 2 (Peak 2)



Compound 3 (Peak 3)

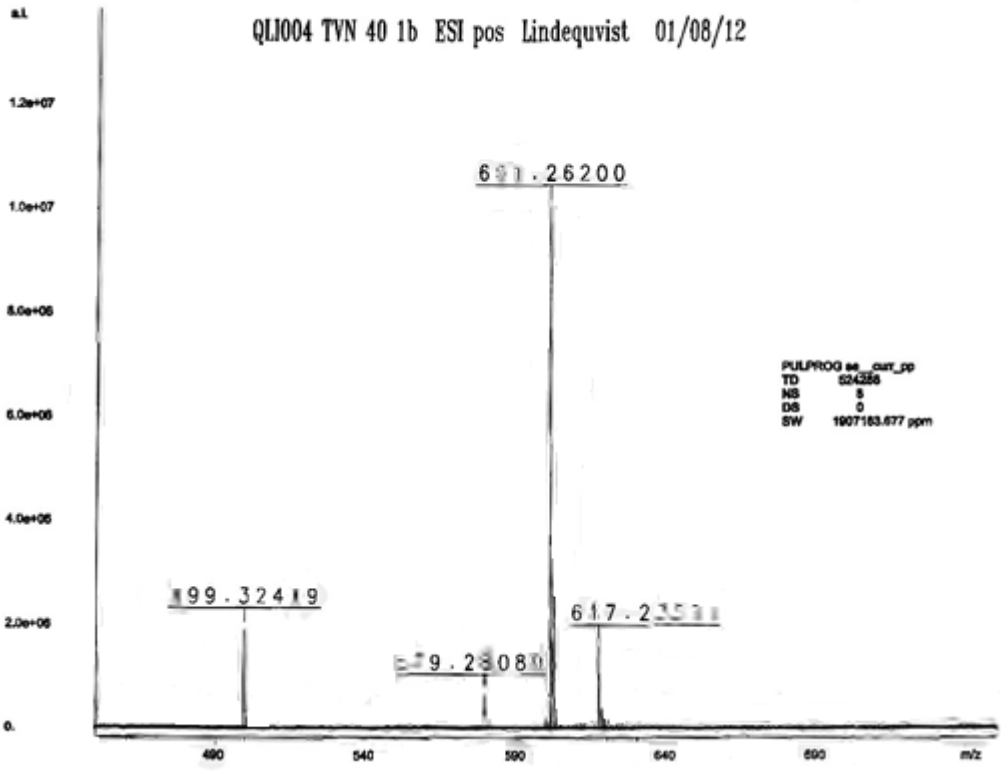


Compound 5 (Peak 5)



VII. Supplementary information of structural elucidation of isolated compounds of TVN40

Compound 1

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H       1     200           0.00   100.00
N       0     6             0.00   100.00
O       0     20            0.00   100.00
Na      0     1             0.00   100.00


#    C     H     N     O     Na     mass          DBE  error     .
*** Mass Analysis for mass 601.2620000
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3    30    39    3     10    0      601.2629959   13.0  1.656e-06
4    31    38    4     7     1      601.2632707   14.5  2.113e-06
5    28    40    3     10    1      601.2605906   10.0  2.344e-06
6    27    41    2     13    0      601.2603158   8.5  2.801e-06
7    32    41    0     11    0      601.2643386   12.5  3.889e-06
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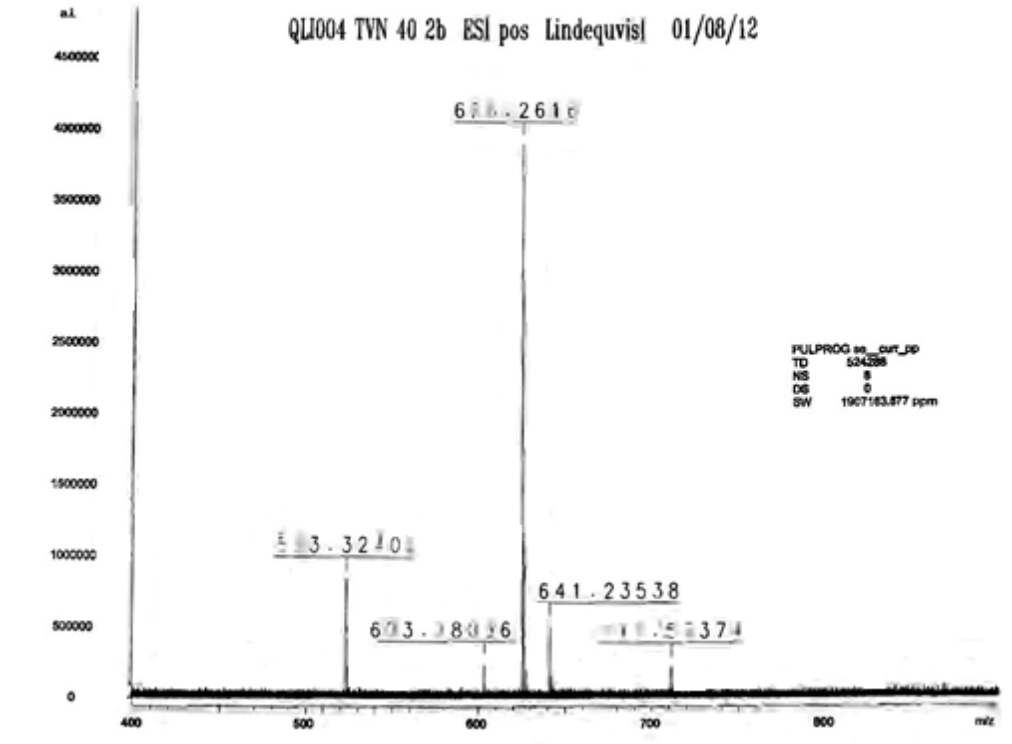
Compound 2

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O        0      20            0.00   100.00
Na       0      1             0.00   100.00


#    C      H      N      O      Na     mass          DBE  error    .
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3    17     45     4      20     0      625.2621663   -2.5  8.898e-07
4    18     44     5      17     1      625.2624411   -1.0  1.329e-06
5    30     40     3      10     1      625.2605906   12.0  1.630e-06
6    29     41     2      13     0      625.2603158   10.5  2.070e-06
7    32     39     3      10     0      625.2629959   15.0  2.217e-06
8    33     38     4      7      1      625.2632707   16.5  2.656e-06
9    20     46     2      18     1      625.2637838   -1.5  3.477e-06
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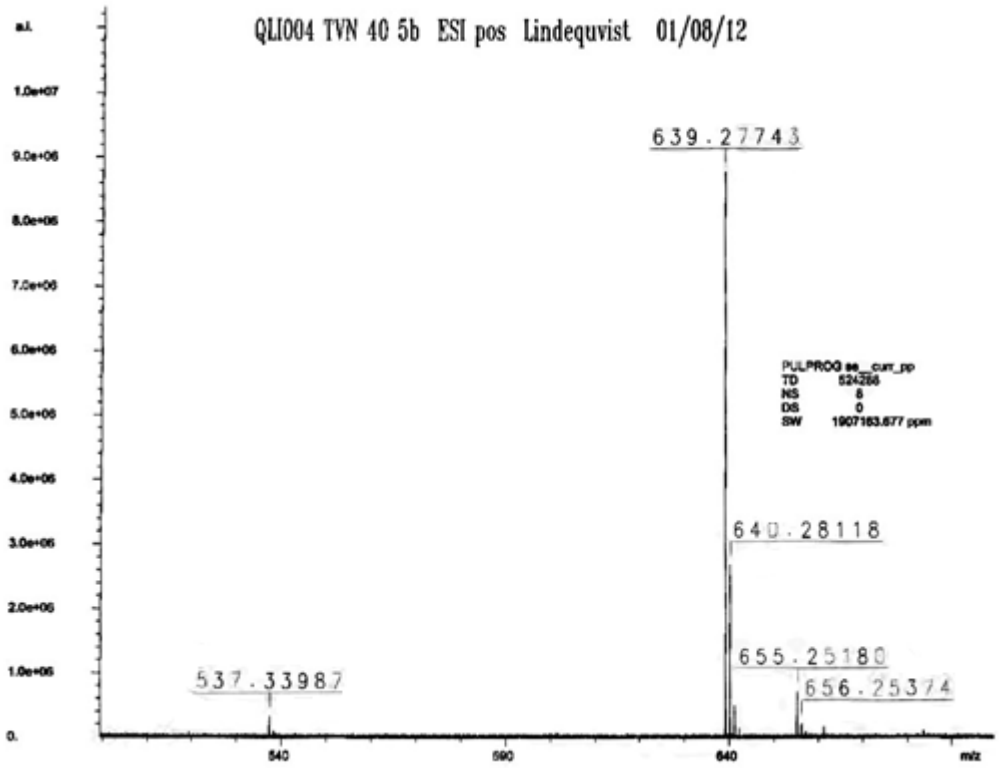
Compound 5

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H        1      200           0.00   100.00
N        0      6             0.00   100.00
O        0      20            0.00   100.00
Na       0      1             0.00   100.00


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3    18     47     4      20     0      639.2778164   -2.5  6.060e-07
4    19     46     5      17     1      639.2780912   -1.0  1.036e-06
5    31     42     3      10     1      639.2762407   12.0  1.859e-06
6    33     41     3      10     0      639.2786460   15.0  1.904e-06
7    30     43     2      13     0      639.2759659   10.5  2.289e-06
8    34     40     4      7      1      639.2789207   16.5  2.333e-06
9    21     48     2      18     1      639.2794338   -1.5  3.136e-06
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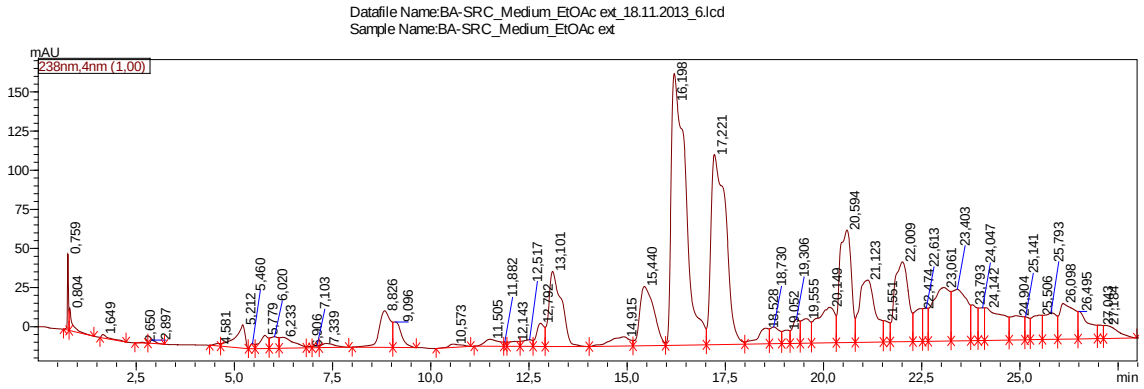
VIII. ESIMS analysis for the presence of flourensadiol

BA-SRC_Medium_EtOAc extract



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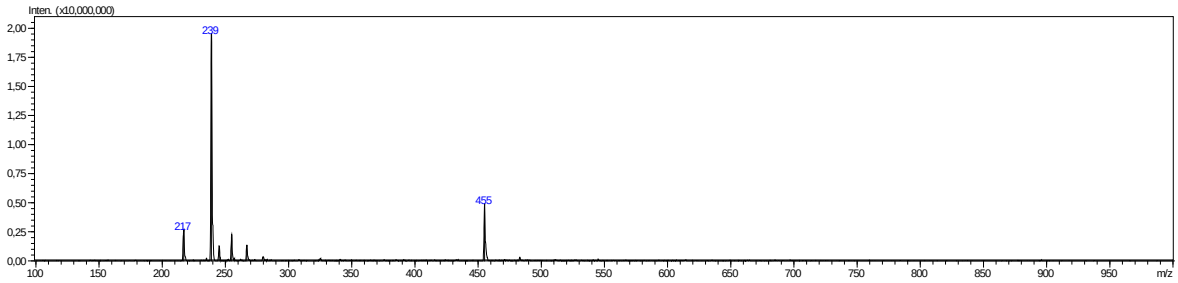

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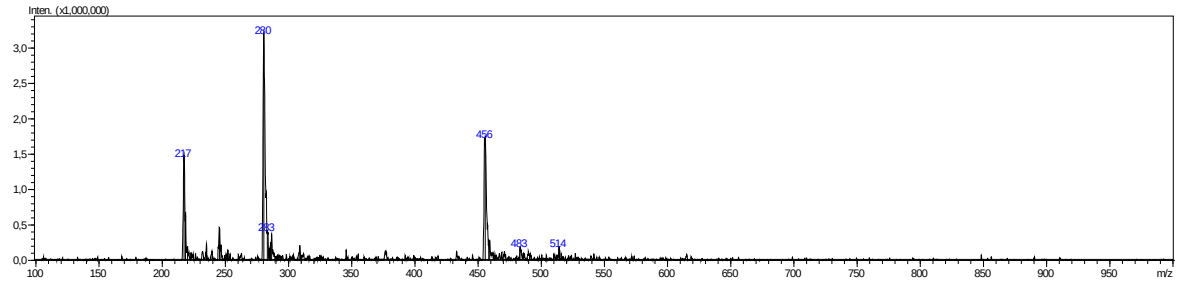

IX. Partial 16S rRNA gene sequences

Bio 33

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TVN40

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WL1

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WS1

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Y1

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YM1

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Erklärung

Hiermit erkläre ich, dass diese Arbeit bisher von mir weder an der Mathematisch-Naturwissenschaftlichen Fakultät der Ernst-Moritz-Arndt-Universität Greifswald noch einer anderen wissenschaftlichen Einrichtung zum Zwecke der Promotion eingereicht wurde.

Ferner erkläre ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die darin angegebenen Hilfsmittel und Hilfen benutzt und keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen habe.

Greifswald, den 04. Juni 2014

Unterschrift des Promovenden

Thanh Huong Bui

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Aug. 2005-Dec. 2008	Lecturer, Department of Chemistry, University of Dalat, Lamdong, Vietnam.
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2000-Aug. 2004	B.Sc. in Chemistry Pedagogics, University of Dalat, Lamdong, Vietnam.
1997-2000	High school in Baoloc, Lamdong, Vietnam.

List of Publications

Publication

- Thanh-Huong Bui, Victor Wray, Manfred Nimtz, Torgils Fossen, Gudrun Schröder, Kristian Wende, Michael Preisitsch, Stefan E. Heiden, Sabine Mundt. Balticidins A-D, Antifungal hassallidin-like Lipopeptides from the Baltic Sea Cyanobacterium *Anabaena cylindrica* Bio 33. Journal of Natural Products, accepted (05. 2014).
- Sabine Mundt, Huong T. Bui, Michael Preisitsch, Susann Kreitlow, Ha T. N. Bui, Hang T. L. Pham, Elmi Zainuddin, Le T. A. Tuyet, Gerold Lukowski, Wolf-Dieter Jülich. Microalgae - a promising source of novel therapeutics. JSM Biotechnology and Biomedical Engineering, Special Issue on "German Industrial Biotechnology", accepted (04. 2014).
- Bui Thanh Huong, Huynh Dinh Dung (2008). Isolation and determination Tetracycline residues in honey using a solid-phase extraction cleanup and UV spectroscopy detection. Scientific announcement of Dalat University, 94 – 101.

Posters

Bui, T. H.; Wray, V.; Nimtz, M.; Fossen, T.; Schröder, G.; Wende, K.; Mundt, S. Four novel antifungal compounds from the Baltic Sea cyanobacterium Bio 33 (2012), DPhG Jahrestagung 2012, Moleküle, Targets und Tabletten – Translationale Forschung für Arzneimittel der Zukunft, Greifswald, Germany.

Thanh-Huong Bui, Gudrun Schröder, Rolf Jansen, Victor Wray, Kristian Wende, Sabine Mundt. (2011), A new antifungal compound from the cyanobacterium *Anabaena* sp., MIMAS-Symposium, Microbial Interations in Marine Systems, Alfried-Krupp-Wissenschaftskolleg Greifswald, Germany.

Thanh-Huong Bui, Gudrun Schröder, Rolf Jansen, Victor Wray, Kristian Wende, Sabine Mundt (2011). A new antifungal compound from the cyanobacterium *Anabaen*a sp., DPhG Doktorandentagung 2011, Heringsdorf, Germany.

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