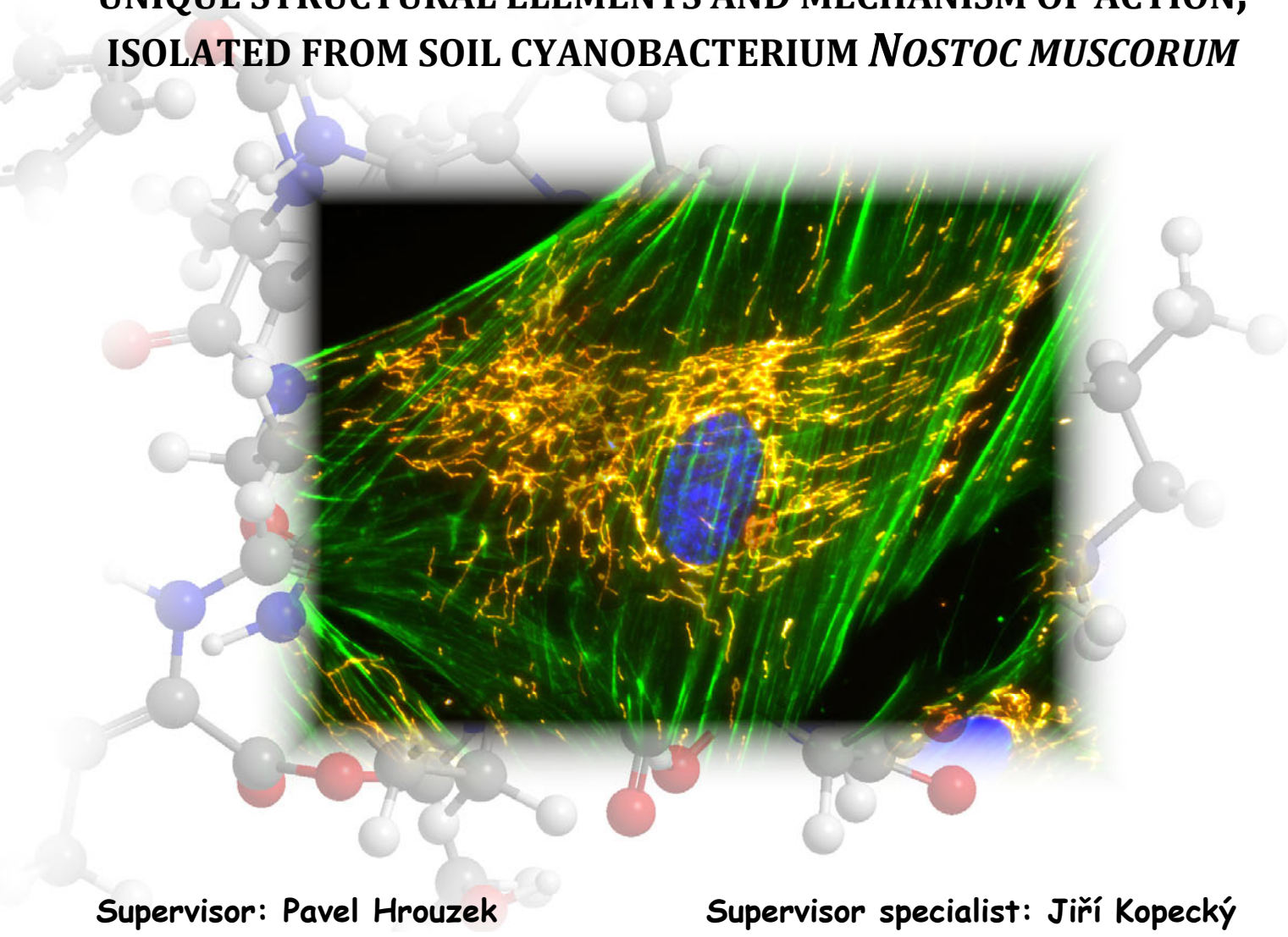
University of South Bohemia

Faculty of Science

Master thesis

Petr Tomek

Muscotoxins: novel cytotoxic undecapeptides with unique structural elements and mechanism of action, isolated from soil cyanobacterium *Nostoc muscorum*



Supervisor: Pavel Hrouzek

Supervisor specialist: Jiří Kopecký

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Annotation:

This project was focused on development of extraction and purification protocol for novel cytotoxic compound isolated from soil cyanobacterium *Nostoc muscorum* which would allow to determine its molecular structure via NMR (nuclear magnetic resonance) and MS (mass spectrometry) techniques. Purified cytotoxin was subjected to several biochemical and microscopical experiments in order to assess its toxicological parameters, determine the mechanism of action and evaluate potential application in biotechnology or pharmacy.

I hereby declare that this master thesis was created solely by myself only by using the sources summarized in the list of cited references.

I affirm that in accord with § 47b of law no. 111/1998 Sb. in its valid version, I agree to publish my thesis by faculty, in its nonreduced form, via electronic means in publicly accessible section of STAG database located on the web page run by The University of South Bohemia.

In České Budějovice, 30.4.2010

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Petr Tomek

Acknowledgement:

Most importantly, I would like to express my undying gratitude to my supervisor Pavel Hrouzek. Our cooperation continued from previous bachelor project and I would say that during the work on this master project we have promoted the relation of supervisor and student to fully-fledged scientific colleagues. We were working as a team, sharing insights, ideas for work and solutions to the emerged problems. Eventually, this collaboration was concluded by a very successful result which will be published as soon as possible. We have conceived many interesting ideas apart from this project and I hope there will be enough time in the future to execute them. Additionally, I´m grateful for his final touch and help during „construction" of this thesis. I have to admit that Pavel was perfect colleague and it is difficult to imagine anyone else on his place.

I would like to thank Jan Cerny from Charles University in Prague for his advices regarding microscopy and cell biology, hospitality whenever I showed myself in his lab. I have to appreciate that despite his rather occupied time schedule he was always able to find some time to reffer about the results or give valuable ideas for further experiments.

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My very important thanks go also to Marek Kuzma and all people from Laboratory of molecular structure characterization at Institute of Microbiology in Prague without whom all my efforts would be worthless. They was the main moving force when assessing the structure of my compounds.

Jan Sykora was wonderful companion when I was working with him in Prague. He provided immense knowledge and equipment background when conducting fascinating experiments on synthetic bilayers.

I would also like to thank the boss of our lab, Jiri Kopecky for his valuable insights regarding chromatography and MS and despite Jirka is rather sceptic person all the time he was able to help when needed and could cheer up the life by ton of jokes and stories from his personal experience. Also I would like to thank Lada Samcova, our technician for her agreeable company and help. But not only her, also to many wonderful women at our institute which I thank even for their presence, let alone pleasurable communication :)

Thus, my thanks go to all friends and colleagues from our institute at Opatovicky Mlyn in Trebon and mainly to drivers of our institute car for comfortable trips to the lab. I thank everybody for cheerful company, and last but not least for the relevant scientific advices when needed. I express my thanks to my parents and whole family who beared with me during hard times.

I would like to convey my apologies to all my friends for neglecting them during recent few months when I was working on this thesis and additionally I also thank myself for being able to get this to a victorious end.

Furthermore I´m deeply sorry for anyone I missed in this summary, please accept my apologies, it was not intentional and I can arrange to fix it every time in person ;)

TABLE OF CONTENT

1. REVIEW

On the forthcoming pages of this opening chapter I am going to give a review of a natural products-derived anticancer drugs, its development with emphasis on cyanobacterial metabolites and their cytotoxins.

Introductory talk about natural products in general is going to be followed by the summary of the molecular targets exploited by natural products in anticancer treatment. Cyanobacteria will be introduced in the next chapter with a focus on their peculiar biosynthetic pathways and a comprehensive up-to-date summary and evaluation of cytotoxic metabolites. The most interesting cytotoxins, either in clinical trials or with promising activities, will be discussed in detail further on. Additionaly, other metabolites of biotechnogical or pharmacological value will find its place at the end of the review concluding the chapter.

1.1. THE NATURAL RESOURCES

Nature is a rich source of unusual metabolites synthesized via an innumerable and many times rather peculiar biosynthetic pathways, which can be used as a powerful means in human medicine and biotechnology (NISBET & MOORE, 1997; HARVEY, 1999; MANN, 2002). This strikingly demonstrates the fact, that around half of the drugs currently in use are of natural origin (PATERSON ET AL., 2005).

One of the most important topics in drug discovery of natural products today is unambiguously anticancer activity (GERWICK ET AL., 2001; NEWMAN ET AL., 2004; MAYER & GUSTAFSON, 2004; CRAGG ET AL., 2005; TAN, 2007; CRAGG ET AL., 2009). Traditional medicine and use of natural products initiated large-scale screening programme of metabolites produced by various organisms by NCI (National Cancer Institute in USA) in 60. – 70. years of previous century (NEWMAN & CRAGG, 2004), which led to discovery of many important structures used in current chemotherapy including famous taxol (WANI ET AL., 1971), podophyllotoxin (GORDALIZA ET AL., 2004), vincristine & vinblastine (JOHNSON ET AL., 1963), camptothecin (WALL ET AL., 1966), bleomycin (RAO ET AL., 1980), epothilone (HOFLE ET AL., 1996), eleutherobine (LINDEL ET AL., 1997), its analogues and many others. Their mechanisms of action have been likewise thoroughly described which made contribution also in fundamental biological science (NISBET & MOORE, 1997).

Although natural products possess high pharmacological potential and specific mechanisms of action, they weren´t synthesized as a systemic anticancer agents, thus they frequently lack optimal parameters desired in pharmaceutically useful drugs. So, it is highly unlikely that purified natural compound could serve directly as anticancer drug in the future.

Fortunately, there are many ways of organic chemistry that can help in tailoring the molecules to the pharmacological needs (reducing toxicity, bestowing drug-like properties) (CRAGG, 2005; NEWMAN ET AL., 2006; KAISER ET AL., 2008). Additionally, it was found out that microorganisms don´t employ its full genomic potential and it is possible to find cryptic

Figure 1.1. Examples of natural combinatorial chemists. **Top Left:** Madagascar periwinkle *Vinca rosea*. (Courtesy of Forest & Kim Starr). **Top middle:** Predator snail *Conus* sp. (Courtesy of Asbjorn Hansen). **Top right:** Squash beetle *Epilachna borealis* (Attribution: Doug Moyer). **Bottom left:** Deepwater octocoral *Eleutherobia grayi*. (Copyright DeepSeaImages /Paul Osmond). **Bottom right:** Nudibranch mollusk *Philinopsis gardineri* (Courtesy of Brian Mayes).

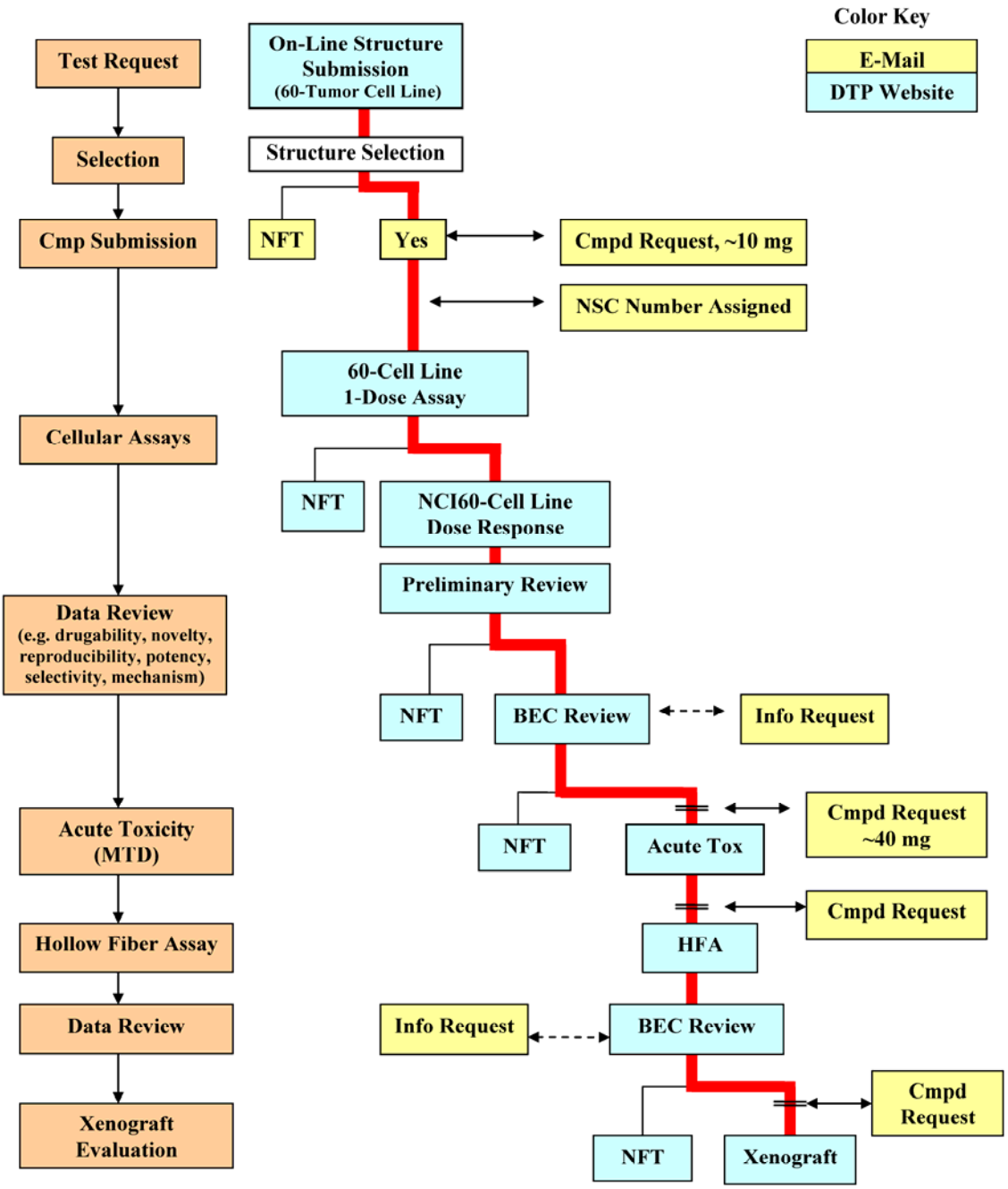
biosynthetic clusters (BENTLEY ET AL., 2002; BOK ET AL., 2006) or genetically modify the actual ones to obtain novel „unnatural" natural products opening much more possibilities in search for optimalized compounds (WALSH ET AL., 2004; CHOU ET AL., 1998; MAGARVEY ET AL., 2006).

Furthermore, natural products are unique and unexpendable leads for the drug development programmes recognized to be an evolutionary privileged structures (EVANS ET AL., 1988).

Complex natural chemical libraries synthesized by organisms need to be well resolved and „potential candidates" perfectly characterised, and that is the task of DTP (Development Therapeutics Programme) markedly demonstrated on Fig. 1.2. During the first stage of screening, there is a need to identify the compounds possessing bioactivity via bioassay-guided fractionations and to evaluate their potency, selectivity, toxicity and mechanisms of action. Second stage stands mainly for thorough *in vivo* investigations before the clinical trials are initiated later.

At first, standard cell line screens are employed to assess antitumour activity. There are many types of biochemical assays to evaluate the proliferation (NAKAYAMA ET AL., 1997) but these approaches possess an adverse drawback – they are too nonspecific.

Modern screens on the other hand are putting emphasis on to be more specific compared to a wide-spectral assays based on pure cytotoxicity. They are utilising transformed cells (OOSTINGH ET AL., 2008), intermediates in biochemical or genetic pathways, a receptor-ligand interactions (SAUTEL & MILLIGAN, 2000) or impedance-based cell assay evaluation (ABASSI ET AL., 2009) and many more (BLAKE, 2001; HERTZBERG & POPE, 2000) which can eventually provide us with much



Abbreviations: **NFT** = No further testing; **BEC** = Biological Evaluation Committee; **MTD** = Maximum tolerated dose; **HFA** = Hollow fiber assay

Figure 1.2. DTP Anti-cancer screening paradigm demonstrating drug development process utilized at NCI in USA. Source: http://dtp.nci.nih.gov/screening.html

more precise detection of bioactive compounds in complex natural matrices including preliminary mode of action elucidation.

The handful of compounds, that have been found to possess novel structures, previously unreported bioactivities or simply good selectivity and potency will make it into preclinical *in vivo* process. Evaluating pharmacokinetics, toxicity and maximum tolerated doses are primary

objectives there. NCI then uses Hollow Fibre Assay to prescreen the potential anticancer candidates before definitive human Tumour Xenograft models.

After the second stage, the compound is advanced into clinical phase, or in case of failure in any step going back as a lead for organic chemists to be either optimized using semisynthetic approaches and then recycled into process, or discarded

1.2. OVERVIEW OF MOLECULAR TARGETS OF NATURAL CYTOTOXINS USED IN ANTICANCER TREATMENT

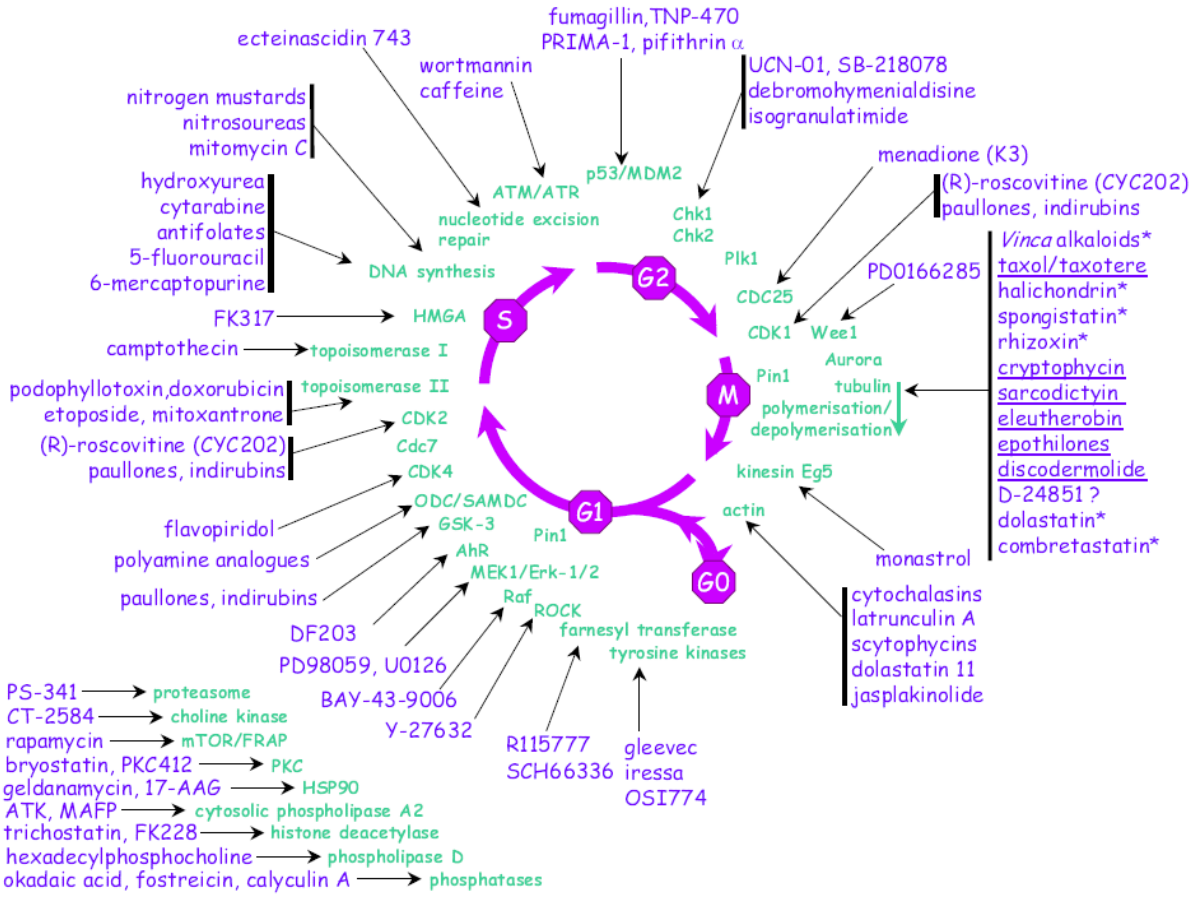


Figure 1.3. Therapeutic agents and their molecular targets. (Used with kind permission from Prof. Laurent Meijer).

Knowledge of MOA (mechanism of action) is essential in developing new anticancer drugs not only concerning preselection the pharmacologically acceptable compounds, but also in prioritizing them for further research and developing optimal treatments. My intention here is not to give a comprehensive list of all possible molecular targets, which would be far beyond the scope of this review, but on the other hand I would like to present and demonstrate the major MOA exhibited by natural products and approaches for their assignment in cancer treatment. The main categories will be as following:

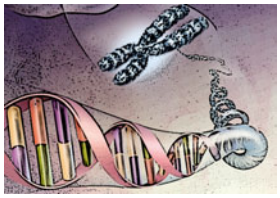
- DNA interacting agents

- Signalling pathways inhibitors (modulators)
- Cytoskeleton disruptors

In addition, there are many miscellaneous targets aiming at modulation of cancer cell resistance (AVENDANO & MENENDEZ, 2002), inhibition of heat-shock proteins (ZHANG & BURROWS, 2004), proteasome function (ADAMS, 2004), angiogenesis (AKHTER ET AL., 1999) and others... . But these are small groups and I will not discuss them in detail. General summary including most important targets and its inhibitors is depicted on Fig. 1.3 originally published by Meijer (MEIJER, 2003).

I will briefly dicuss every category, including fundamental principles and few examples of clinically used agents (focused exclusively on natural anticancer chemotherapeutics). For more details including another compounds, specific applications or detailed MOA, readers are reffered to comprehensive reviews published e.g. by Thurston or Avendano (THURSTON, 2007; AVENDANO & MENÉNDEZ, 2008), which also provided the main scaffold and if not stated otherwise, also majority of discussion for the upcoming part.

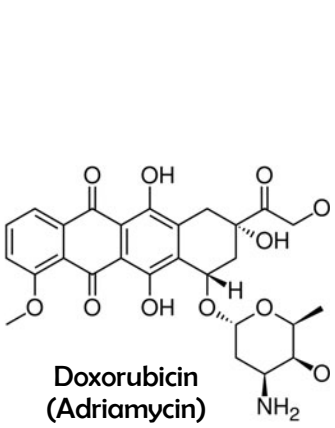
1.2.1. DNA IMPAIRING COMPOUNDS



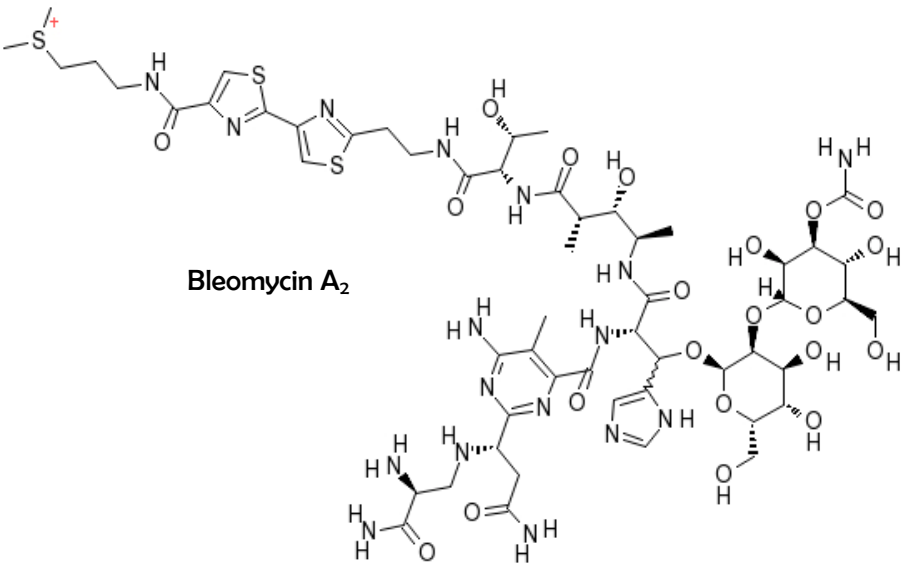
1.2.1.1. ACTING THROUGH ROS (REACTIVE OXYGEN SPECIES) FORMATION

Molecular oxygen, or its singlet, superoxide, peroxide species or hydroxyl radicals are all oxidative agents, chemical individuals with an unpaired electron, generally known as ROS generated in any actively metabolizing cell (ANDREYEV ET AL., 2005). The drugs belonging to this category are not damaging the cell explicitly. They are rather promoting formation of such molecules, which in turn induce oxidative stress (imbalance between antioxidants/ROS) in the cell and cause harm to biomolecules (lipids, proteins, DNA).

Cellular membranes with their polyunsaturated fatty acid carbon chains are highly susceptible to damage caused by ROS, mainly hydroxyl radicals, which results in phospholipid peroxidation chain reaction and membrane injury. Many times, esterification of polyunsaturated fatty acids leads to forming of another dangerous product - MDA (malondialdehyde). Ultimately causing cross-linking of protein aminogroups and DNA damage by reacting with guanine amino groups modifying them into pyrimido-purine derivatives. Such obstructions on DNA results usually in mitotic arrest. ROS can also be responsible for direct damage to DNA by oxidation of bases resulting in block of replication or increasing reading errors of replication machinery. Additionally, many other dangerous compounds can be generated by action of radicals, like for example formaldehyde etc... (AVENDANO & MENENDEZ, 2008).



Doxorubicin
(Adriamycin)



Bleomycin A_2

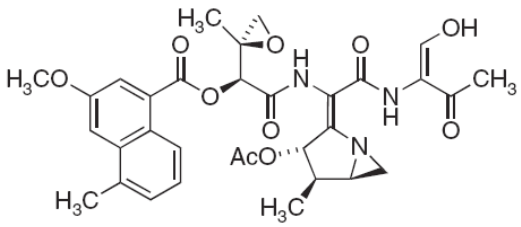
Most striking example in this category of drugs are anthracyclines and their congeners being represented notably by famous and widely used anticancer agents doxorubicin and daunorubicin (FREDERICK ET AL., 1990). Another notable examples are bleomycins (RAO ET AL., 1980), natural complex glycopeptide antibiotics produced by *Streptomyces verticillus*.

1.2.1.2. DIRECTLY DAMAGING ALKYLATING AGENTS

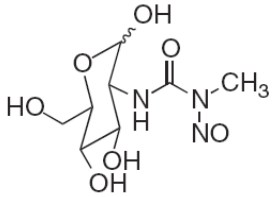
This category includes many nonnatural-derived structures including famous nitrogen mustards (derived from war gas yperite designed by Nazis), aziridines, methanesulfonates, triazines (initally developed as a resin precursors) or well-known platinum complexes (serendipitously discovered during experiments with electric current effect on cells), but several are also natural-products-derived, like in the cases of epoxides or nitrosoureas.

Epoxide ring is very reactive towards negatively charged parts of biomolecules, thus also effective DNA alkylating agents. Carzinophilin, aziridine-epoxy bacterial metabolite (FUJIWARA ET AL., 1999), is striking example of that exhibiting cytotoxic activities at nanomolar concentrations.

Nitrosoureas, first discovered by NCI are lipophilic agents able to cross blood-brain barrier, thus useful in treating brain tumours, but their major problem is relatively high toxicity. Diminishment of toxicity was introduced by natural product streptozotocin (REUSSER ET AL., 1971), isolated from *Streptomyces achromogenes* which served also as scaffold for many analogues still used in modern chemotherapy.



Carzinophilin



Streptozotocin

1.2.1.3. DNA MINOR GROOVE INTERACTING AGENTS, INTERCALATORS AND TOPOISOMERASE INHIBITORS

There are two basic categories of compounds in this group. Intercalators and minor-groove binders (MGB). Chemotherapeutics in this group are striking example of SAR behaviour.

Structures of MGBs can be recognized by simple aromatic ring systems connected by torsionally free bonds in order to efficiently complement the minor groove (hydrogen bonds, sterical factors...) and they are either reversibly binding or alkylating DNA. On the other hand, intercalators are planar aromatic compounds inserting itself in between the bases causing distortion in helical conformation. Such structures most commonly inhibit DNA and RNA polymerases and other DNA associated proteins like transcription factors, repair systems and most importantly, the topoisomerases.

Topoisomerases are important enzymes for reducing supercoiling stress and optimizing topology during DNA replication and transcription, and thus predisposed targets for anticancer drugs. There are two main types, topoisomerase I (breaking single DNA strand) and topisomerase II (breaking both DNA strands consuming ATP) (WANG ET AL., 1991). The drugs focused at these targets are either inhibiting its primary catalytic function or interfering with transesterification rejoining step, thus converting topoisomerases into „DNA-killers“. Imperative to say, that inhibiting topoisomerases is one of the most efficient ways to induce apoptosis, so these compounds (mainly of the latter category) belong to one of the clinically most effective anticancer agents in use.

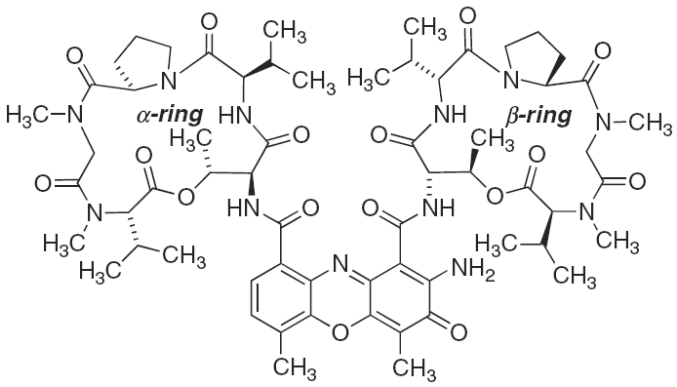
Best example of topoisomerase I inhibitors are already mentioned camptothecins which underwent many chemical modifications in the past and results are worth of acclaim. Many compounds are in clinical trials, for example elomotecan. They form a very stable complex with cleavable topoisomerase I (II)-DNA union which makes them forsooth effective (LAVERGNE ET AL., 1999).

On the other hand, etoposide, synthetic derivate of natural microtubule inhibitor podophyllotoxin, is capable inhibitor of topoisomerase II (HARTMANN, 2006). This plant-metabolite acts specifically during S and G2 phase of cell cycle and prevents release of hydrolysed ADP from topoisomerase II complex.

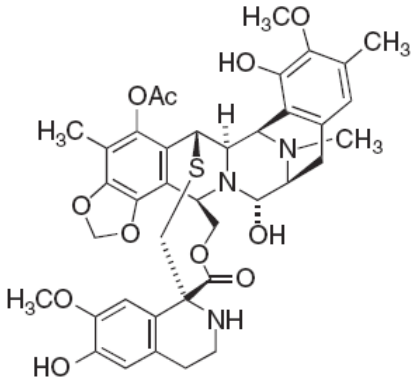
Due to development of resistance againts one type of inhibitors in cancer cells there is high interest in compounds that can inhibit both enzymes or substituting it by co-treatment with both topoisomerase I & II inhibitors at low concentrations resulting in synergy.

Typical example of MGB is natural product distamycin A (RAJALAKSHMI ET AL., 1978) and its synthetic derivative brostallicin (TEN TIJE ET AL., 2003) recognizing A-T rich sequences in minor groove and alkylating DNA, or tetrahydroisoquinoline alkaloids saframycin S and ecteinascidin (RINEHART ET AL., 1990). Ecteinascidin 743 is particulary interesting MGB exhibiting highest potency from the group and currently in phase III clinical trials displaying intriguing MOA. Its binding to the minor groove cause distortion of the helix and thus activation of nucleotide excision repair mechanism normally cutting out damaged part of molecule and resynthesizing it by polymerase. But in this unique case, repair is reversed and endonucleases damage the DNA instead by lethal single-strand breaks (TAKEBAYASHI ET AL., 2001).

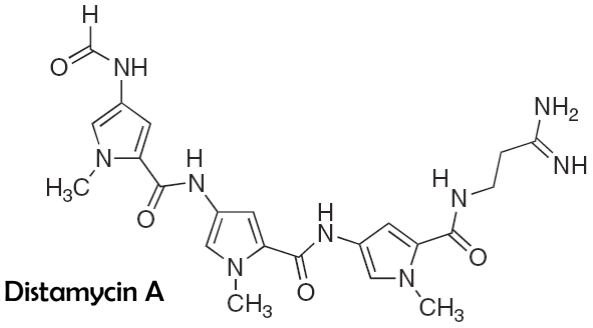
Concerning intercalators, actinomycin D is noteworthy compound. This bacterial cyclic bisdepsipeptide-phenoxazine is actually one of the most potent intercalators due to its hybrid mode of action interacting both with major and minor groove which results in excellent stability and high potency (SOBELL ET AL., 1985). It can be classified as a monofunctional intercalator as opposed to bi(poly) functional ones, which distort DNA at more than one site like another natural product echinomycin, potent bisquinoxaline-octadepsipeptide. Two chromophores in such structure are resposible for intercalation at two proximal sites stabilized by central peptide ring (HUANG ET AL., 1982).



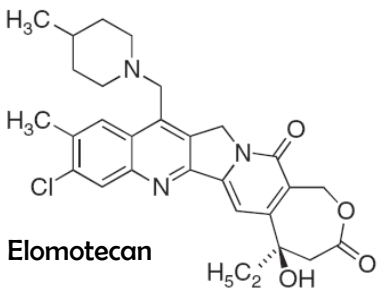
Actinomycin D



Ecteinascidin - 743

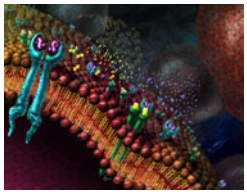


Distamycin A



Elomotecan

1.2.2. SIGNALLING PATHWAYS INHIBITORS



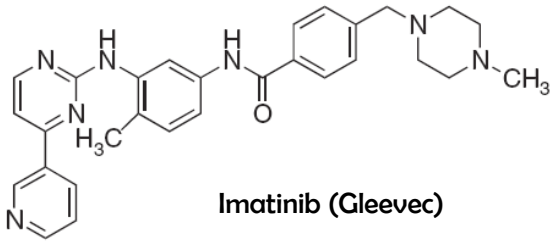
Disturbances in communication has usually profound consequences resulting in pathological disorders. Cells need to know what to do, where to migrate, when to divide, when to rest, when to die, and they have to constatnly maintain contact with others, thus there is a need for tight regulation of these „activities“. On intracellular scale that control is provided by special enzymes called protein kinases, which typically phosphorylate (activate) other enzymes responsible for crucial cell processes like proliferation an differentiation.

Roughly about 20% of all our genes accounts for proteins regulating signal transduction and many cancer cells have overexpressed key protein kinases regulating proliferation and therefore they are susceptible targets for cancer chemotherapy. Several protein kinases have been recognised as pharmaceutic targets for cytotoxins acting mainly by interaction with enzyme ATP-binding site.

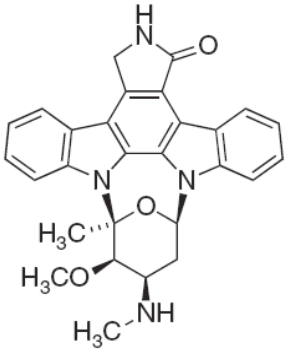
There are three main types of protein kinases classified according to the amino acid where the phosphorylation takes place, namely Ser/Thr (serine/threonine), Tyr (tyrosine) and His (histidine) protein kinases. Altough very few natural compounds act as kinase inhibitors I consider this section worthwhile to mention, due to its potential in cancer treatment.

1.2.2.1. TYR KINASES INHIBITORS

These kinases are represented mainly by membrane bound receptor types responsible for reception and transduction of growth signals like for example EGFR (epidermal growth factor receptor), VEGFR (vascular endothelial growth factor receptor) or Abelson kinase (mutated BCR-ABL kinase responsible for Chronic myaeloid leukemia). Many synthetic inhibitors (small molecules, antibodies) have been designed mimicking ATP or tyrosine substrates.



Imatinib (Gleevec)



Staurosporine

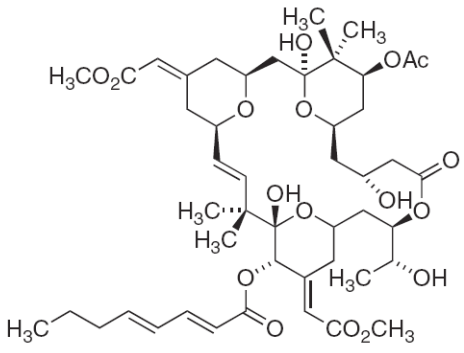
Although it is not a natural drug, I would like to mention imatinib (Gleevec), which revolutionized treatment of CML by specific targeting of mutated BCR-ABL kinase present in diseased cells (CAPDEVILLE ET AL., 2002). Concerning natural products, staurosporine (OMURA ET AL., 1977) is potent non-selective kinase inhibitor, altough its lack of selectivity precluded it from clinical use. On the other hand, bis-indole alkaloid scaffold served as a template for semisynthetic approach resulting in many analogs, e.g. CEP-5214 being selective VEGFR inhibitor in clinical trials (GINGRICH ET AL., 2003).

1.2.2.2. SER/THR KINASES INHIBITORS

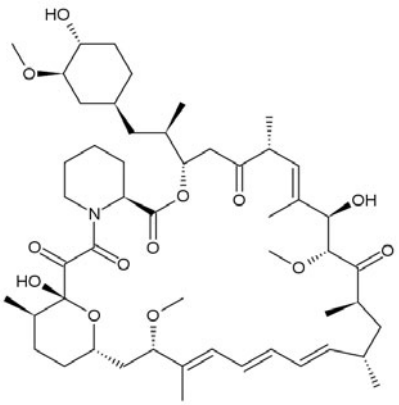
Kinases included in this group are mainly cytoplasmic enzymes regulating cell-cycle progression (cyclin dependent kinases – CDKs); translation, transcription, angiogenesis, apoptosis (PI3K-AKT-mTOR cascade) or important proliferation regulators (PKC).

Rapamycin, relatively simple macrolide isolated from *Streptomyces hygroscopicus* (SEHGAL ET AL., 1975), is inhibitor of mTOR (mammalian Target Of Rapamycin) kinase regulating translation of proliferation- and survival-specific genes. Being upregulated in tumours, it is perfect target for therapy. Rapamycin is in clinical trials.

Cyclic macrolide bryostatin 1 (PETTIT ET AL., 1982A), on the other hand is responsible for modulating activity of PKC and very promising agent, because PKC provides input into MAPK (mitogen activated protein kinases) pathway (HALE ET AL., 2002). This is one of the most complex signalling cascade crucial for promoting of cell division, which connects extracellular signal reception with its intracellular transduction. This impressive molecule suffers from serious supply issue which hampers its clinical trials but several simpler analogues have been already synthesized (WENDER ET AL., 2002).



Bryostatin 1



Rapamycin

1.2.2.3. APOPTIC SIGNALLING PATHWAYS MODULATORS

Apoptosis, or programmed cell death, is a highly organized self-determined process of cell death necessary for maintaining cellular homeostasis, successful development, proper function of immune system or destruction of pathogen-infected cells (NELSON & WHITE, 2004).

Main regulators of the apoptic process are caspases, cystein proteases cleaving its targets after aspartate residues. They exist normally as an inactive zymogens, proteolytically cleaved (activated) by various activators, usually initiator caspases and later even by itself amplifying the death sentence. There are two main ways leading to its activation, the stress-induced (intrinsic) or death ligand-induced (extrinsic) pathway. Both usually converges downstream activating effector caspases -3,-6 and -7 (EARNSHAW ET AL., 1999).

Extrinsic pathway in humans starts by receiving death ligand (e.g. CD95L) at one of the death receptors (CD95). Lipid raft diffusion caused by generation of ceramide results in clustering of activated receptors into DISC (Death Inducing Signalling Complex) revealing death domain on the inner side of membrane. It attracts adaptor protein FADD (Fas-associated death domain) which in turn recruits procaspases -8 or -10 allowing them to autocatalyze and ignite chain reaction resulting in activation of executor caspases (HENGARTNER, 2000) (Fig. 1.4).

The more ancient intrinsic (stress-related) pathway is mediated by cytochrome c release from disrupted mitochondria. This „cell-native cytotoxin", usually serving as a humble electron carrier, induces conformational change in adaptor proteins Apaf-1 which are then able to attract and bind procaspase-9, oligomerize into huge heptamer called apoptosome catalyzing its substrate into active caspase-9. This molecular juggernaut (~1 MDa) launches massive amplification of signal including activation of caspases -3 and -7 and DNAses responsible for chromatin break-down (HENGARTNER, 2000). Altough apoptosome drastically enhances the rate of apoptosis, it is not essential component for its completion (MARSDEN ET AL., 2002).

Executor caspases-3 and -6 have many substrates and they are potent destructors, inhibitors and activators in one. There are three major hallmarks of caspase-mediated apoptosis.

1) **Activation:** Cleavage of DNA into nucleosomal units. Altough caspases are not explicitly responsible for it, they activate CAD (caspase-activated DNase) by cleaving its inhibitor subunit.
2) **Inhibition:** Compromising DNA repair mechanisms. Amongst many, notable is PARP poly (ADP-ribose) polymerase, important repair enzyme and a substrate for inhibition by caspase-3.
3) **Destruction:** Breakdown of structural and other proteins. Most importantly, caspases disintegrate nuclear lamins resulting in nuclear shrinking (caspase-7), cytoskeletal proteins like keratins, actin, gelsolin responsible for cell structural integrity, many protein kinases, proinflammatory cytokines and many other targets (ELLIS & HORVITZ, 1986; EARNSHAW ET AL., 1999).

Additionally, there are several factors regulating the apoptosis pathways, like tumour suppresing p53 factor or Bcl-2 class proteins (LEVINE ET AL., 1994). Bcl-2 class of proteins contains both pro- (e.g. Bax, Bid) and anti- (e.g. Bcl-2) apoptic proteins and their interplay control the cell susceptibility to apoptosis (CORY & ADAMS., 2002). Bcl-2 proteins act as a cellular stress sensors and when they „feel" the stimulus, they relocate to mitochondrial membrane and interaction with pro-apoptic proteins can result in mitochondrial pore opening (Permeability Transition pore) releasing cytochrome c and other regulating factors like antiapoptic SMAC/Diablo or proapoptic

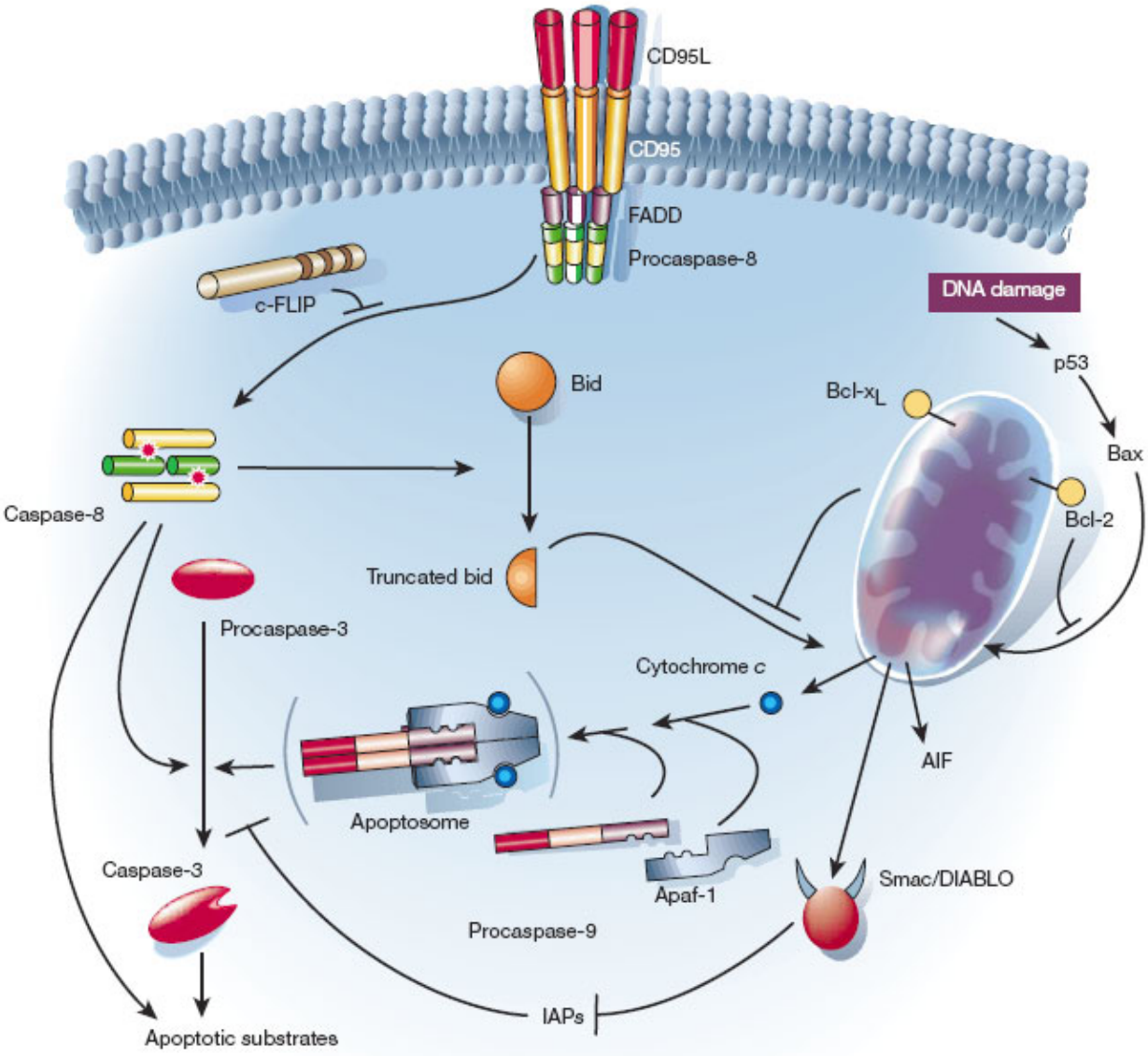


Figure 1.4. The roads to ruin: Two major apoptic pathways in mammalian cells.
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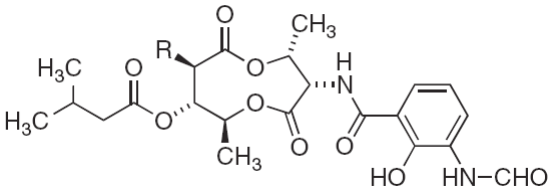
AIF (Apoptosis Inducing Factor), flavoprotein, which induces very potent, but not well understood caspases-independent cell death (HENGARTNER, 2000). Indeed, mitochondria pack a lot of „firepower“ and its role in apoptosis is of high importance and acts as an intricate „input/output hub“ of signals. Exception proves the rule and apoptosis can also proceed without mitochondrial contribution as it is in the case of e.g. extrinsic stimulus (HENGARTNER, 2000) (Fig. 1.4).

Ultimately, this elaborate process neatly dismantles the cell to membrane-enclosed apoptic bodies, displaying „eat-me“ signals in the form of externalized phosphatydilserine, which alerts phagocytes in the proximal vicinity readily engulfing them to prevent release of its intracellular contents to the microenvironment (SAVILL & FADOK, 2000).

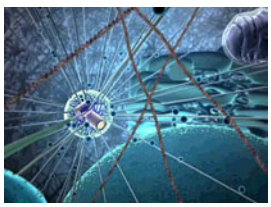
As the deregulation of apoptosis is the key element in every genuine cancer cell (HANAHAN & WEINBERG, 2000), it can also seriously hampers the efficiency of chemotherapeutic agents. Indeed, anti-apoptic Bcl-2 proteins are overexpressed in many tumours and p53 factor inactivated. Thus, there is a great deal of efforts to reverse the apoptic functions back to normal in tumours. Example of such approach is a natural product antimycin A successfully inhibiting Bcl-2 protein (TZUNG ET AL., 2001), thus rendering the cancer cells more susceptible to apoptosis. Other promising treatments directed at apoptosis signalling include introduction of function copy of TP53 into p53-defective cells or directly inducing extrinsic apoptosis pathway via TRAIL/DR4 ligand/death receptor able to be effective even in tumours with mutated Bcl-2 (KONDOH ET AL., 2004; KASIBHATLA ET AL. 2005).



Antimycin A

But sometimes, initial insult result in a drastic events, not as subtle as apoptosis, that is when necrotic cell death is induced. Altough necrosis is not so well defined as apoptosis, there are some major differences that allow us to identify it. Necrosis is a result of total bioenergetic collapse (ATP, nutrient or oxygen deprivation), swelling of cellular organelles and loss of membrane integrities, followed by a release of immuno-stimulating and reparative factors (ZONG & THOMPSON, 2006). As opposed to apoptic death, where cell need to maintain its integrity to proceed with energetically demanding processes, orderly degradation of chromatin and release of immuno-suppresive factors (KIECHLE & ZHANG, 2002). Even when it seems that there is a clear boundary between these types of cell death, actually there is not and they usually share same stimuli and many agents are able to induce both apoptosis and necrosis in concentration-dependent manner, thus there is a possibility to find features of both death modes in one dying cell (ZONG & THOMPSON, 2006). For example when cell can´t be engulfed by phagocytosis e.g. in *in vitro* culture, late apoptic cells eventually succumb to necrosis (MAJNO & JORIS, 1995).

1.2.3. TUBULIN AND MICROTUBULES INTERACTING DRUGS („MITOTIC POISONS“)



It is probably the best known chemotherapeutic target of many natural compounds and that is also why I will go into more deatail in this section. It is proposed that downstream pathways related to tubulin binding are apoptosis-inducing operators in malignant cells (PELLEGRINI, ET AL, 2005).This is the most widespectral group of anticancer agents used clinically for treating various types of both haematological and solid cancers for many decades (HADFIELD ET

AL., 2003; HAIT ET AL., 2007). Their function as „cell-killers" is still unchallenged due to the nature of highly susceptible and polar tubulin/microtubules assembly and its responsibility for many crucial intracellular processes such as cell division, maintenance of cell integrity, intracellular topology layout (localization of nucleus and other organelles) and serving as a modular „highway" for motor proteins carrying important cargos (organelles, chromosomes etc.) (PELLEGRINI, ET AL., 2005). Counted up an underscored, microtubules are unexpandable and also abundant. The protein surface area of microtubules accounts for the same as the cytoplasmic membrane (GUNDERSEN & COOK, 1999).

Structurally, microtubules consist of 13 linear protofilaments comprised of non-covalently bound tubulin (made of two similar α– and β– protein subunits with GTP binding domains) forming hollow cylinder. In order to respond to the cell requirements swiftly, microtubules maintain many forms of stable/unstable arrays. Phenomenon known as dynamic instability regulated by several factors including innate ability to hydrolyse GTP at tubulin β-subunit, propelling so-called treadmilling effect of depolymerisation at –end and rapid polymerisation at +end of microtubule. Thus, we can propose changes in the equilibrium of tubulin/microtubules cause usually fatal problems. Cell can recruit many regulatory or stabilizing factors in order to „tame" the equilibrium either in advantage of assembled microtubules (e.g. tau) or tubulin subunits (e.g. katanin) (HADFIELD ET AL., 2003).

Fundamentally, the cytotoxins interacting with microtubules induce reversible/irreversible changes to this equilibrium resulting mainly in mitotic arrest, but additionally also disruption of many other miscellaneous pathways. Altough these drugs are used for a long time, their antitumour action has not been fully understood, especially the connection between mitotic arrest and cell death (PELLEGRINI ET AL., 2005).

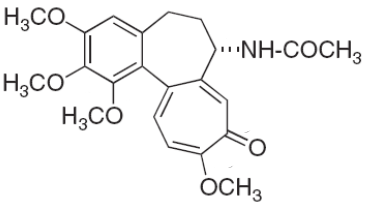
We can divide anti-mitotic agents into compounds interacting either with tubulin or with MAPs. Tubulin interacting agents can be further divided into several categories according to the binding site on the subunit named usually after its best known ligand:

- Colchicine binding site
- Vinca alkaloids binding site
- Taxanes binding site
- Other/unknown binding site
- KSP (Eg5) inhibitors

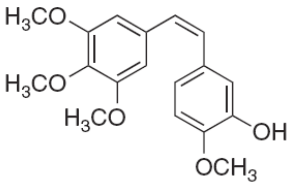
1.2.3.1. COLCHICINE BINDING SITE

Compounds in this group binds to the tubulin at intra-dimer interface and modifies lateral contacts within microtubules, which results in depolymerization at high concentrations or stabilization of dynamics at low concentrations (HADFIELD ET AL., 2003). Best known example is natural tropolone alkaloid colchicine, one of the first investigated antimitotic agent binding to tubulin irreversibly. Unfortunately, its high toxicity never allowed it to enter clinical medicine (JORDAN ET AL., 1998). Common pharmacophore in colchicine binding compounds is

trimethoxyphenyl group which is present in another notable compound – combretastatin A-4, natural product isolated from african willow *Combretum caffrum* (PETTIT ET AL., 1995). Alhtough compound is very effective in binding to tubulin its main problem is low solubility which led to synthesizing many promising analogues (HADFIELD ET AL., 2003).



Colchicine



Combretastatin A-4

1.2.3.2. VINCA ALKALOIDS BINDING SITE

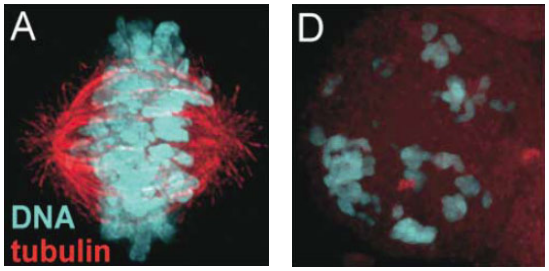


Figure 1.5. Spindle defects produced by antimitotic drug treatments. **A:** The normal bipolar spindle of an untreated cell at metaphase. **D:** Vinblastine depolymerizes microtubules, preventing spindle assembly.

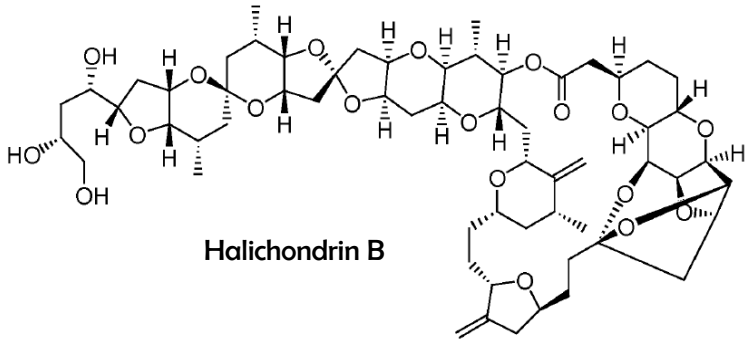
This category gathers structures that are binding to tubulin at inter-dimer section causing spatial distortion (higher affinity for each other) and thus i nhibiting microtubule assembly (HADFIELD ET AL., 2003). Together with colchicine binding site agents they are considered microtubule depolymerizers and same concentration-dependent behaviour as with colchicine binding agents apply even here.

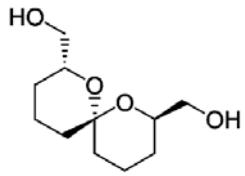
Members of this class are, already mentioned, so-called v*inca* alkaloids (according to the plant they were isolated from – *Vinca rosea* (periwinkle)), complex metabolites introduced into chemotherapy in late 60´s of previous century and still important structures in clinical treatment. They are represented mainly by vinblastine and vincristine and few semisynthetic analogs. Altough still routinely used, they possess serious flaws hampering their superior anticancer assignment, notably neurotoxicity and easy development of drug resistance.

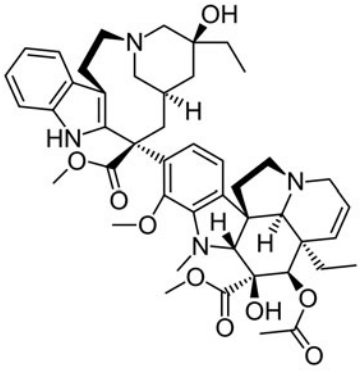
One of the better alternatives are metabolites halichondrins and spongistatins. But there are many other effective compounds, for example cyanobacterial peptides cryptophycins or dolastatins which I will talk about later.

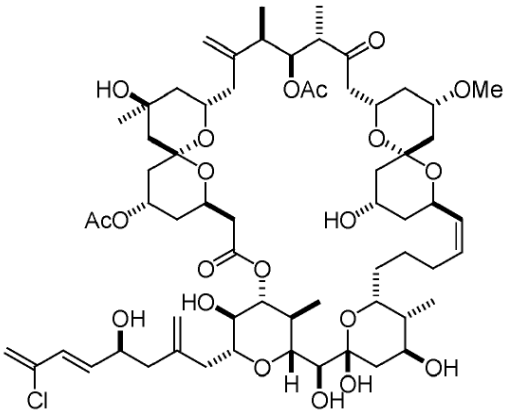
Halichondrin B was isolated from several species of marine sponge (HIRATA & UEMURA., 1986). This polyether macrolide possesses unprecedentedly high therapeutic index. To promote it to clinical trials, SAR experiments allowed to identify pharmacophore and synthesize less complex molecules synthetically opening its way to clinical trials (Analogue E7389) (NEWMAN & CRAGG, 2004).

Spongistatin 1 is another sponge metabolite, this time macrocyclic lactone with interesting spiroketal moieties displaying extreme cytotoxic potency in pM concentrations and it is now examined in clinical trials (BAI ET AL., 1993). Additionally, tubulin binding studies revealed that compound binds to different site in *Vinca* domain, which led to rational development of simple, highly effective SPIKET compound containing mere single spiroketal system (UCKUN ET AL., 2000B).









1.2.3.3. TAXANES BINDING SITE

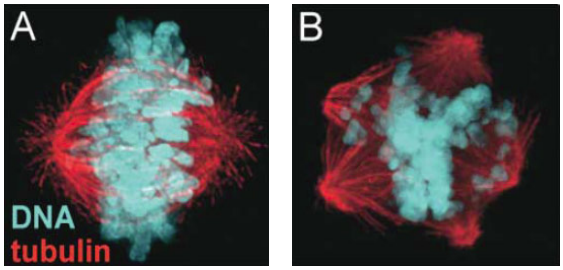


Figure 1.6. Spindle defects produced by antimitotic drug treatments. **A:** The normal bipolar spindle of an untreated cell at metaphase. **B:** Taxol-stabilized microtubules produce multipolar spindles.

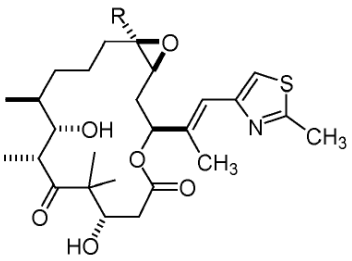
Unlike the tubulin depolymerizers, this distinct group is chracterised by binding to M-loop of β-tubulin, stabilising interactions between individual monomers in microtubule protofilaments (SCHIFF ET AL., 1979).

Primary ligand, the famous paclitaxel, is paradoxically a victim of the same fate as the *vinca* alkaloids impairing its non-problematic therapeutic use. Original molecule suffers from poor solubility and easy development of resistance in tumours. These adverse traits were partially eliminated by semisynthetic approach resulting in many analogs, e.g. ortataxel (orally active), but the coveted aim is far from reality (GENEY ET AL., 2005).

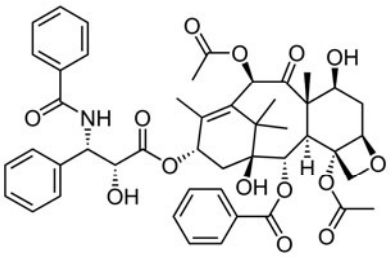
Epothilones A and B are relatively simple macrolides displaying higher potency, better aqueous solubility and mainly they are poor substrates for drug resistance mechanisms. Its major drawback are metabolic lability of lactone rings which were made more stable in semisynthetic ixabepilone now undergoing clinical trials (GOODIN, 2008).

Discodermolide, polyketide metabolite isolated from sponge *Discodermia dissoluta*, is another broadspectral cytotoxin effective against many taxol and epothilone resistant cell lines (TER HAAR

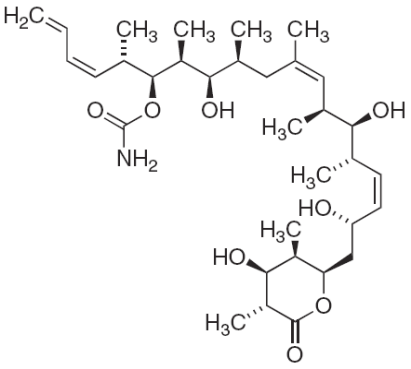
ET AL., 1996). It is also one of the scarce natural products which total synthesis were developed (SMITH ET AL., 2008).



Epothilone A



Paclitaxel



Discodermolide

1.2.3.4. OTHER/UNKNOWN BINDING SITE

Compounds included in this class act on other than previously mentioned tubulin binding sites. There are curerntly no natural products in this group, but lets mention at least rationally designed cobra compounds acting at rather unique leucine rich α-tubulin domain (UCKUN ET AL., 2000A).

1.2.3.5. MOTOR PROTEINS INHIBITORS

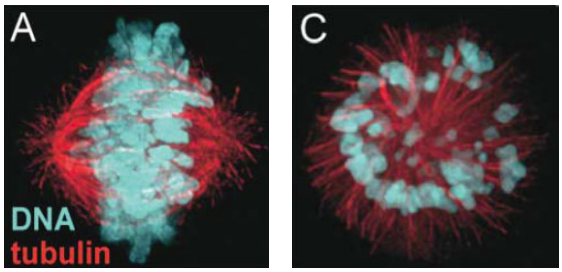
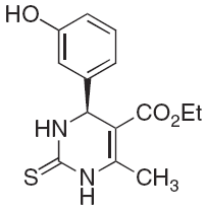


Figure 1.7. Spindle defects produced by antimitotic drug treatments. **A:** The normal bipolar spindle of an untreated cell at metaphase. **C:** Monastrol, a KSP/Eg5 inhibitor, prevents spindle pole separation, resulting in monopolar spindles. All figures in this section reprinted from Cancer Cell, Vol. 8/Issue 1, Beth A.A. Weaver, Don W. Cleveland, Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death, Pages 7-12, Copyright 2005, with permission from Elsevier.

Up to this point, every anti-mitotic compound mentioned were interacting with only one protein – tubulin. It is peculiar, because there is a high abundance of various spindle formation proteins that could serve as other potential targets, so why nature developed only agents binding to tubulin remains an unanswered question (AVENDANO & MENÉNDEZ, 2008).

One of them are definitely kinesins, motor proteins responsible e.g. for moving chromosomes along the microtubules. Eg5 was identified as an attractive target for spindle assembly inhibition (TAO ET AL., 2005). It is very promising and this approach could have one notable advantage over other agents and that is selectivity only for proliferating



Monastrol

tumour cells theoretically avoiding adverse neurotoxicity in other terminally differentiated cells like neurons connected with other anti-mitotic drugs. Few drugs have been synthesized so far like e.g. monastrol (DEBONIS ET AL., 2003) and ispinesib (LAD ET AL., 2008).

Because the chemotherapeutic drugs inflicts severe damage even to normal healthy cells, there are much efforts invested into the research dealing with targeting the drugs specifically to tumour tissues. Indeed, this is an intricate objective, but many successes have been achieved so far.

One of the most developed field is prodrug-based therapy based on delivering inactive prodrugs that are selectively activated in tumour tissues exploiting specific attributes of its physiology (selective enzyme expression, hypoxia etc..). Enzymes responsible for activation of prodrug are usually delivered either in the form of expressible genes on vector (GDEPT - gene-directed enzyme prodrug therapy), virus (VDEPT - virus-directed enzyme prodrug therapy) or conjugated with antibodies (ADEPT - gene-directed enzyme prodrug therapy) (AVENDANO & MENÉNDEZ, 2008).

Other important group is based on passive delivery of macro- or nano-particles into interstitial tissue of tumours, which is known for its greater permeability and disorganized state where the particles can be captured. Systems used in this type of therapy are for example macromolecule-drug conjugates, PEG-treated liposomes or albumin bound nanoparticles <100nm (AVENDANO & MENÉNDEZ, 2008).

1.3. CYANOBACTERIAL SECONDARY METABOLITES

These microscopic organisms have existed on our planet for at least 2 milliard years and they have been responsible for two key points in shaping the environment as we know it now. Firstly, oxygenating the Earth´s atmosphere and secondly, delivering the ability of photosynthesis to plants via endosymbiosis (SIVONEN, 2009; GARCIA-PICHEL, 2009), both allowing evolution of aerobic organisms and sustained source of oxygen.

They are able to survive and flourish practically everywhere on our planet except from acidic environments (RASTOGI & SINHA, 2009). To achieve such adaptability and resiliency nature granted them several powerful attributes (THAJUDDIN & SUBRAMANIAN, 2005) from which most spectacular is ability to synthesize incountable array of secondary metabolites. Truly, cyanobacteria play an important role as a promising, remarkable source of novel compounds of pharmacological and biotechnological significance (CARMICHAEL, 1992; NAMIKOSHI & RINEHART, 1996; BURJA ET AL., 2001; GERWICK ET AL., 2001; THUJADDIN & SUBRAMANIAN, 2005; TAN, 2007; RASTOGI & SINHA, 2009).

The majority of these metabolites is small molecules of appx. 150-2000 Da produced via intricate multienzyme complexes reffered to as NRPS (non-ribosomal peptide synthetase) (WELKER & VON DÖHREN, 2006) and PKS (poly-ketide synthetase) (STAUNTON & WEISSMAN, 2001) which could be best described as an extensively modular factory assembly lines (VAN WAGONER ET AL., 2007) which composition and sequence is predetermined on a genetic level forming a gene clusters (WASE & WRIGHT, 2008). Their major features are nonstandard and unique forms of amino

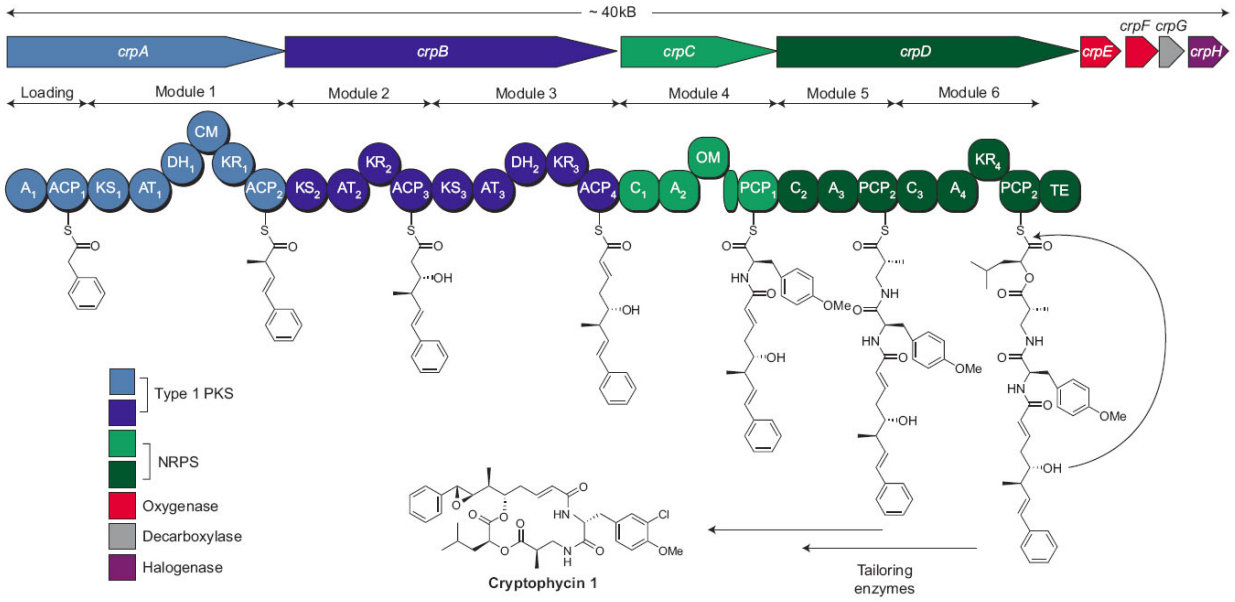


Figure 1.8. The cryptophycin gene cluster and the biosynthetic assembly line process. Domains of the PKS and NRPS multienzyme complexes: A = adenylation domain, ACP = acyl carrier protein, KS = β-keto synthase, AT = acyl transferase, DH = dehydrase, CM = C-methyl transferase, KR = ketoreductase, C = condensation domain, OM = O-methyl transferase, PCP = peptidyl carrier protein, and TE = thioesterase.
Reprinted from ACS Chemical Biology, Vol 1 /Issue 12, Jürgen Rohr, Cryptophycin Anticancer Drugs Revisited, Pages 747-750, Copyright 2006, with permission from ACS.

acids and hydroxy acids also combined together with fatty acids and carbohydrates.

Altough both systems are significantly different according to their structure and biosynthesis, they share the flexibility and core principle which many times allow their genes to be mixed and produce hybrid synthetases (VAN WAGONER ET AL., 2007). Such complex enzyme systems are able to deliver unique hybrid entities in many cases exclusive for cyanobacteria (GERWICK ET AL., 2001). Striking demonstration is depicted on Fig. 1.8 in the case of NRPS/PKS of cyclic hybrid depsipeptide crypthophycin (ROHR, 2006).

Considering the enormous potential of both individual systems, such a combination underscores seemingly infinite amount of structures hatching from these interesting machineries.

Thanks to all those features and structural peculiarities, namely e.g. presence of β- or hydroxy- amino acid moieties (STEER ET AL., 2002) or N-methylated peptide bonds (MEDINA ET AL., 2008), these compounds can be highly resistant to proteolysis and thus readily bioavailable in treated organism increasing both potency and effect duration when compared to a „standard" bioactive compounds. (DEMUYS ET AL., 1996; LUESCH ET AL., 2001C; ANDRIANASOLO ET AL., 2007; MEDINA ET AL., 2008)

There are hundreds of cyanobacterial compounds described so far which are proposed to be only a fraction of a real world diversity (HROUZEK ET AL., 2010). Plenty of them possesses some kind of bioactivity or unique property making them fascinating targets for research, but at the same time it is very challenging to bring some order in to these enormously variable metabolites according to some rules. In many studies authors are either discussing these products from pharmacological (NAMIKOSHI & RINEHART, 1996; BURJA ET AL., 2001; TAN, 2007), biotechnological or structural diversity perspective (VAN WAGONER ET AL., 2007; RASTOGI & SINHA, 2009), or they are

focusing on the dangers of these products for public health, their geographic distribution and the role in the nature (CODD, 2005; BLAHA ET AL., 2009; HROUZEK ET AL., 2010).

The following lines will be dedicated to cyanobacterial cytotoxins and their use in pharmacy, and other pharmacologically or biotechnologically interesting compounds.

1.3.1. CYANOBACTERIAL CYTOTOXINS

It is the most abundant group of cyanobacterial metabolites, it is estimated that 30% of cyanobacterial strains posses cytotoxic metabolites (HROUZEK ET AL.,2010) . In the last three decades, hundreds of cytotoxins were isolated and identified from cyanobacteria originating from distincts regions and habitats in the world (prevalently marine environment) and several of them have become strikingly promising leads for anticancer applications surpassed by only a handful of other natural products yet (MAYER & GUSTAFSON, 2004; TAN, 2007).

On the following pages Im going to present a comprehensive list of discovered cyanobacterial cytotoxins published in literature in years 1999-2009. Then I will discuss the results and pick promising compounds either in clinical trials or ones displaying novelties and interesting MOAs. The table encompasses 82 alphabetically ordered cytotoxins/cytotoxin classes (~ 240 in total including congeners) with data about its chemistry, molecular weight, organism of origin, IC_{50}. For the sake of this chapter, to avoid any discrepancies, the term cytotoxin will be considered as a compound with general ability to kill mammalian cancer cells (exluding all primary hepatotoxic, neurotoxic etc. compounds).

I have drawn the information from individual scientific reports as well as few reviews released in last decade, which guided me to structures discovered even before the year 1999 rendering the ultimate coverage even more comprehensive.

The structural classification in my table is based on a review published by van Wagoner and colleagues (VAN WAGONER ET AL., 2007) with minor changes and of course I will be listing only categories which contains cytotoxins. Following structural types are covered:

Polyketides

These compounds are synthesized solely by PKS enzymes from various small acids (acetate, propionate,...) and chacharacteristic by absence of nitrogen atom. But many times, halogens are incorporated, structures are cyclised or miscellaneous subunits attached being able to produce rather exotic molecular entities.

- Long-chain alcohols
- Cyclic Polyketides
- Aromatic polyketides
- Lactones

Peptides

Peptides is the most plentiful group and majority of cytotoxins belongs to this category. They can be synthesized via sole NRPS systems resulting in simple linear or cyclic peptides, in which aminoacids (or other subunits) are connected by amide bonds, or depsipeptides, characterized by substitution of at least one amide bond by ester bond which most of the times cyclise the molecule resulting in cyclic macrolactone peptide (cyclic depsipeptide). Or alternatively they can be produced via hybrid PKS/NRPS introducing polyketide subunits into peptide molecules or vice versa, thus all variants of peptides can theoretically contain PKS-derived unit resulting in hybrid products.

- Linear & cyclic /hybrid/ peptides
- Linear & cyclic /hybrid/ depsipeptides

Alkaloids

This group is characterized by introducing a nitrogenous subunit into polyketide, isoprenoid or shikimate precursors containing usually single amide link. As it is typical for cyanobacterial secondary metabolites, the alkaloids can cyclise, contain lactones or various aromatic- or hetero-cycles.

- Lyngbic acid alkaloids
- Macrolide alkaloids
- Small heterocyclic alkaloids
- Indole alkaloids
- Aromatic alkaloids
- Nucleoside analogues

Table 1.1. Summary of cytotoxins isolated from cyanobacterial sources

PI[A]	Compound[B]	Genus[C]	Chemistry	MW (Da)	IC_{50} (ng/ml)[D]	Reference
12	9-deazaadenosine, (5´-α-D-Glucopyranoside-)	*Anabaena*	nucleoside analogue	266-428	2 – 10	NAMIKOSHI ET AL., 1993
43	Acutiphycin	*Oscillatoria*	pyran macrolide	480	500	BARCHI ET AL., 1983
25	Ankaraholides A-B	*Geitlerinema*	macrocyclic glycosylated bislactone	1680-1694	15 – 442	ANDRIANASOLO ET AL., 2005
7	Aplyronines A-C	*Aplysia k.* (sea hare)	eneamide macrolide alkaloid	945-1075	0.48 – 21.2	KIGOSHI ET AL., 1996; YAMADA ET AL., 1993
4	Apratoxins A-E	*Lyngbya*	cyclic hybrid depsipeptide	795-881	0.3 – 57.2	LUESCH ET AL., 2001C, 2002D; MATTHEW ET AL., 2008; GUTIÉRREZ ET AL., 2008
32	Aulosirazole	*Aulosira*	quinone alkaloid	261	45 – 350	STRATMANN ET AL., 1994
19	Aurilides A-C	*Lyngbya*	cyclic hybrid depsipeptide	819-833	8.3 – 106.4	SUENAGA ET AL., 2004; HAN ET AL., 2006
36	Aurisides A-B	*Dolabella a.* (sea hare)	complex glycosidic macrolide	673-804	170 – 1200	SONE ET AL., 1996
28	Bauerines A-C	*Dichothrix*	indole alkaloid	216-266	30 – 5000	LARSEN ET AL., 1994
41	Belamide A	*Symploca*	linear peptide	642	475	SIMMONS ET AL., 2006
31	Bisebromoamide	*Lyngbya*	linear hybrid peptide	1021	40	TERUYA ET AL., 2009A
34	Biselyngbyaside	*Lyngbya*	glycosidic macrolide	604	100	TERUYA ET AL., 2009B

33	Borophycin	*Nostoc*	macrocyclic complex lactone	854	66 – 3300	HEMSCHEIDT ET AL., 1994
22	Calothrixins A-B	*Calothrix*	indolocarbazole alkaloid	298-314	12.6 – 104.3	RICKARDS ET AL., 1999; CHEN ET AL., 2003
52	Carbamidocyclophanes A-E	*Nostoc*	macrocyclic aromatic polyketide	671-809	900 – 5100	BUI ET AL., 2007
73	Caylobolide A	*Lyngbya*	macrolactone	763	7500	MACMILLAN & MOLINSKI, 2002
20	Coibamide A	*Leptolyngbya*	cyclic depsipeptide	1287	9.7 – 20000	MEDINA ET AL., 2008
1	Cryptophycins 1-54	*Nostoc*	cyclic hybrid depsipeptide	590-688	0.006 – 31	GOLAKOTI ET AL., 1996; SUBBARAJU, 1997
15	Curacins A-D	*Lyngbya, Symploca*	small heterocyclic alkaloid	373	3.4 – 75	GERWICK ET AL., 1994; YOO & GERWICK, 1995; MÁRQUEZ ET AL., 1998
62	Cylindrocyclophanes A-F	*Cylindrospermum*	macrocyclic aromatic polyketide	568-636	2000 – 10000	MOORE B. ET AL., 1992; SMITH ET AL., 2001
54	Dolabelide A-D	*Dolabella a.* (sea hare)	polyketide macrolide	712-796	1000 – 6000	OJIKA ET AL., 1995; SUENAGA ET AL., 1997
72	Dolabellin	*Dolabella* (sea hare)	linear hybrid depsipeptide	610	6100	SONE ET AL., 1995
2	Dolastatins C,H,10,15,18	*Lyngbya, Symploca*	linear (hybrid/depsi) peptide	620-836	0.02 – 17000	PONCET ET AL., 1998; FLAHIVE & SRIRANGAM, 2005
11	Dolastatins D-G,11-14,16-17	*Lyngbya, Symploca*	cyclic (hybrid/depsi) peptide	487-995	1.9 – 22000	FLAHIVE & SRIRANGAM, 2005
9	Doliculide	*Dolabella a.* (sea hare)	cyclic hybrid depsipeptide	615	1	ISHIWATA ET AL., 1994
58	Grassypeptolide	*Lyngbya*	cyclic hybrid depsipeptide	1102	1102 – 4630	KWAN ET AL., 2008
48	Guamamide	*Symploca*	linear hybrid peptide	487	584.4	WILLIAMS ET AL., 2003
74	Guineamides B-C	*Lyngbya*	cyclic hybrid depsipeptide	627-640	9600 – 10000	TAN ET AL., 2003
27	Hantupeptin A	*Lyngbya*	cyclic hybrid depsipeptide	736	23 – 2950	TRIPATHI ET AL., 2009
66	Hapalosin	*Hapalosiphon*	cyclic hybrid depsipeptide	489	2445 – 7335	STRATMANN ET AL., 1994
23	Hectochlorin, (deacetyl-)	*Lyngbya*	cyclic hybrid depsipeptide	622-664	13.3 – 199	MARQUEZ ET AL., 2002; SUNTORNCHASHWEJ ET AL., 2005
51	Hermitamides A-B	*Lyngbya*	lyngbic acid alkaloid	359-398	790 – 2190	TAN ET AL., 2000
79	Hormothamnin A	*Hormothamnion*	cyclic hybrid peptide	1196	N/A	GERWICK ET AL., 1992
17	Hormothamnione, (6-desmethoxy-)	*Hormothamnion*	aromatic polyketide	370-400	4.6 – 1000	GERWICK, 1989; GERWICK ET AL., 1986
69	Itralamide B	*Lyngbya*	cyclic hybrid depsipeptide	796	4700	JIMENEZ ET AL., 2009
16	Kulokekahilides -1-2	*Philinopsis* (mollusk)	cyclic hybrid depsipeptide	825-955	3.5 – 2100	NAKAO ET AL., 2004; KIMURA ET AL., 2002
26	Largazole	*Symploca*	cyclic hybrid depsipeptide	622	16 – 8000	TAORI ET AL., 2008
60	Laxaphycins B1-B3	*Lyngbya*	cyclic hybrid peptide	1412-1605	1650 – 6300	BONNARD ET AL., 2007
75	Lyngbouilloside	*Lyngbya*	complex glycosidic macrolide	584	10000	TAN ET AL., 2002
29	Lyngbyabellins A-D	*Lyngbya*	cyclic hybrid depsipeptide	895	30 – 500	LUESCH ET AL., 2000 A,B; WILLIAMS ET AL., 2003A
67	Lyngbyaloside B	*Lyngbya*	complex glycosidic macrolide	648	2800 – 9800	LUESCH ET AL., 2002C
24	Lyngbyastatins 1-3	*Lyngbya*	cyclic hybrid depsipeptide	999-1059	14 – 500	LUESCH ET AL., 1999; HARRIGAN ET AL., 1998; WILLIAMS ET AL., 2003B
14	Majusculamide C, (desmethoxy-)	*Lyngbya*	cyclic hybrid depsipeptide	954-984	3.2 – 510	PETTIT ET AL., 2008; SIMMONS ET AL., 2009
38	Majusculamide D, (deoxy-)	*Lyngbya*	linear hybrid peptide	795-811	200	MOORE R. & ENTZEROTH, 1988
3	Malevamide D	*Symploca*	linear hybrid depsipeptide	732	0.2 – 0.5	HORGEN ET AL., 2002

63	Malyngamides O,S	*Lyngbya*	lyngbic acid alkaloid	471-485	2000 – 3900	GALLIMORE ET AL., 2000; APPLETON ET AL., 2002
77	Malyngolide dimer	*Lyngbya*	small δ-bislactone	540	19000	GUTIÉRREZ ET AL., 2010
59	Microcyclamide	*Microcystis*	cyclic peptide	583	1200	ISHIDA ET AL., 2000
39	Micromide	*Symploca*	linear hybrid peptide	903	234.8	WILLIAMS ET AL., 2003
80	Mirabazoles B,C	*Scytonema*	linear peptide	424-438	N/A	CARMELI ET AL., 1991A
55	Mirabilene A-F isonitriles	*Scytonema*	long-chain isonitrile alcohol	407-465	1000 – 10000	CARMELI ET AL., 1990B
70	Mirabimides A-D	*Scytonema*	linear depsipeptide	606-648	5000 – 10000	CARMELI ET AL., 1991B; PAIK ET AL., 1994
45	Mitsoamide	*Geitlerinema*	linear hybrid peptide	1093	502	ANDRIANASOLO ET AL., 2007
71	Nakienone A	*Synechocystis*	small-ring polyketide	194	5000 – 20000	NAGLE & GERWICK, 1995
78	Nakitriol	*Synechocystis*	small-ring polyketide	192	20000	NAGLE & GERWICK, 1995
56	Nostocyclopeptides A1-A2	*Nostoc*	cyclic peptide	757-791	1000	GOLAKOTI ET AL., 2001
44	Nostocyclophanes A-D	*Nostoc*	macrocyclic aromatic polyketide	652-976	500 – 2000	CHEN ET AL. 1991
57	Nostopeptolides A1-A3	*Nostoc*	cyclic hybrid depsipeptide	1066-1080	1000	GOLAKOTI ET AL., 2000
76	Nostophycin	*Nostoc*	cyclic hybrid peptide	888	10000	FUJII ET AL., 1999
47	Obyanamide	*Lyngbya*	cyclic hybrid depsipeptide	599	580	WILLIAMS ET AL., 2002A
68	Pahayokolide A	*Lyngbya*	cyclic hybrid peptide	1472	3135 – 4810	BERRY ET AL., 2004
21	Palau´amide	*Lyngbya*	cyclic hybrid depsipeptide	851	11	WILLIAMS ET AL., 2003E
35	Palau´imide	*Lyngbya*	linear hybrid peptide	428	154 – 600	LUESCH ET AL., 2002A
64	Patellamides A-F	*Prochloron*	cyclic peptide	742-776	2000 – 10000	MOHAMMAD ET AL., 1995; IRELAND ET AL., 1982
61	Pitipeptolides A-B	*Lyngbya*	cyclic hybrid depsipeptide	807-809	1950 – 2250	LUESCH ET AL., 2001B
10	Scytophycins A-E	*Scytonema, Cylindrospermum*	eneamide macrolide alkaloid	805-821	1 – 100	ISHIBASHI ET AL., 1986; JUNG ET AL., 1991
13	Somocystinamide A	*Lyngbya, Schizothrix*	linear hybrid peptide dimer	758	2.2 – 4400	NOGLE & GERWICK, 2002; WRASIDLO ET AL., 2008
8	Swinholides A-I	*Symploca*	macrocyclic bislactone	1388-1416	0.52 – 1401	YOUSSEF ET AL., 2006
30	Symplocamide A	*Symploca*	cyclic hybrid depsipeptide	1051	30.5 – 42	LININGTON ET AL., 2008
5	Symplostatin 1,3	*Symploca*	linear hybrid peptide	746-798	0.3 – 7.7	LUESCH ET AL., 2002E; HARRIGAN ET AL., 1998
81	Tantazoles A,B,F,I	*Scytonema*	linear peptide	564-578	N/A	CARMELI ET AL., 1990A
42	Tasiamides A-B	*Symploca*	linear hybrid peptide	829-979	480 – 3470	WILLIAMS ET AL., 2002B; WILLIAMS ET AL., 2003D
49	Tasipeptins A-B	*Symploca*	cyclic depsipeptide	770-869	631 – 808	WILLIAMS ET AL., 2003C
82	Tjipanazoles A-J	*Tolypothrix*	indole alkaloid	290-485	N/A	BONJOUKLIAN ET AL., 1991
6	Tolytoxin	*Tolypothrix, Scytonema*	eneamide macrolide alkaloid	849	0.44 – 7.1	JUNG ET AL, 1991; PATTERSON ET AL., 1992
18	Toyocamycin	*Tolypothrix*	Nucleoside analogue	279	6	BERGSTROM ET AL., 1984; DE CLERCQ ET AL., 1987
40	Tubercidin	*Tolypothrix*	Nucleoside analogue	254	400	BERGSTROM ET AL., 1984; DE CLERCQ ET AL., 1987, BARCHI ET AL., 1983
53	Tychonamides A-B	*Tychonema*	cyclic hybrid peptide	1456-1486	900 – 3300	MEHNER ET AL., 2008
50	Ulongamides A-E	*Lyngbya*	cyclic hybrid depsipeptide	627-691	659 – 3259	LUESCH ET AL., 2002B
46	Ulongapeptin	*Lyngbya*	cyclic hybrid depsipeptide	808	509	WILLIAMS ET AL., 2003F
65	Westiellamide	*Westiellopsis*	cyclic peptide	452	2000	PRINSEP ET AL., 1992
37	Wewakpeptins A-D	*Lyngbya*	cyclic hybrid depsipeptide	983-1007	196 – 10730	HAN ET AL., 2005

[A] Rather confusing column „PI“ reffers to „Potency Index“, pseudovalue I have designed in order to facilitate the interpretation of gathered data. In order to obtain PI the compounds were sorted separately from lowest to highest according to its best IC_{50} and allocated the numbers in the range of 1-83 ultimately reflecting the degree of potency of every single one. As compounds are arranged in alphabetical order for the ease of search, the PI value brings another perspective of evaluation for the reader, being able to swiftly tell the relative potency of given cytotoxin in the

group. For example compound with PI42 can be without hesitation considered as moderately cytotoxic as opposed to the toxin with PI1 being the most potent one. Additionally, the PI serves as a compound identifier in graphical plot.

B The trivial names of compounds usualy reflects the place of isolation, organism of origin or interesting feature in the preffix and sometimes part of the chemistry in the suffix, thus even if I haven´t included geographical data, many times it is possible to tell the place of isolation, like e.g. palau´amide (Palau), curacin (Curaçao), apratoxin (Apra Bay). and of course ther are references in the last column where the reader can easily find the missing information. Additionally, described congeners can vary in the bioactivity and, so if it is the case I have mentioned only cytotoxic variants (e.g. malyngamides, malevamides, majusculamides etc.).

C Altough sources of several of the listed compounds (e.g. kulokekahilides) are not cyanobacterial, there are sufficient proofs that these toxins can not be produced by invertebrates itself, but rather by ingested cyanobacteria as a dietary source. Unfortunately, in many cases, cyanobacterial genera have not been reliably identified yet. Thus, I´m listing only noncyanobacterial compounds that are considered to be of undirect cyanobacterial origin.

D Only cytotoxins with IC_{50} <20 mg/ml are mentioned in the list, because there is no therapeutical reason to consider metabolites exceeding this value. (even values in mg/ml ranges are considered low). The majority of IC_{50} values were determined using KB (epidermal cancer) and LoVo (colon cancer) cell lines, but that is not true for all compounds, thus the data are not unified methodically (using same cell lines). Unfortunately, in such a various set of resources it is nearly impossible task to perfectly unify the results so I´m well aware that comparison can be a little misleading but regarding the intention of this review there are no major errors present that would significantly untune the outcome. When there is a range of IC_{50} values present in the table it accounts for minimal and maximal values obtained either from all cell lines tested if one compound is concerned (e.g. aulosirazole, belamide A), or from all cell lines and various congeners together as a group result (e.g. dolastatins, calothrixins). In the first case, it is even possible to tell (according to the span of values > 1-2 orders of magnitude) whether the cytotoxic tumour is compound selective, thus potentially attractive for clinical application (e.g. coibamide). Imperative to say that promising antitumor agent can´t be selected solely on its IC_{50} value, but it is a good starting point and benchmark of potency. There are many more clinically important attributes like the compound´s mechanism of action, solubility, selectivity for tumour tissues, pharmacokinetics, bioavailability or therapeutic index.

1.3.1.1. EVALUATION OF CYTOTOXINS

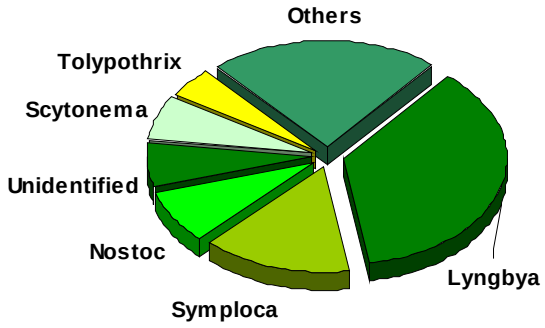


Fig. 1.9. Distribution of sources of cyanobacterial cytotoxins

Majority of 82 cytotoxins originating from cyanobacteria were isolated from *Lyngbya* (35%) and *Symploca* (15%) strains. They are followed by rather small fractions of compounds isolated from *Nostoc* (8%), *Scytonema* (7%), *Tolypothrix* (5%) and cyanobacteria of nonspecified genera marked as unidentified sources (7%). Nearly one quarter (23%) was found to be produced by various genera, not discussed in detail. (Fig. 1.9). Cyanobacterial genera *Lyngbya* and *Symploca* are prolific sources of many cytotoxins, but unfortunately it is impossible to tell whether these organisms could really be the true prominent source, because majority of research was done solely on these two strains living in tropical marine habitats inflicting handicap to other groups. Nevertheless, it is imperative to say that the most potent cytotoxin cryptophycin was isolated from terrestrial cyanobacteria (TRIMURTULU ET AL., 1994) proving these sources could hold more potential if not neglected.

About two-thirds (65%) of reviewed cytotoxins are peptides and the rest is polyketides (21%) and alkaloids (14%) (Fig. 1.10). Such a result markedly reflects the chemotype

distribution of not only cytotoxins, but all cyanobacterial secondary metabolites. Indeed, peptides account for the most abundant and important group of cytotoxins due to their immense structural variabilities and resulting biological specifities for various cellular targets. Up to this date, only a handful of alkaloids or polyketides (curacin A) have been considered as a promising structures for anticancer treatment as opposed to peptides bearing much more potential for this purpose. Carrying out an in-depth analysis of cytotoxic peptides opens up a way to much more interesting results.

After thorough investigation of Fig. 1.10 we can reveal their real hallmark. It is the combination of peptidic backbone with polyketide subunit or vice versa resulting in so called hybrid entities apparently possessing cytotoxic activities more often. Indeed, 78% of all cytotoxins are hybrid molecules mostly synthesized in cyclic forms and as depsipeptides (cyclic hybrid depsipeptides abundance = 43%). The probable explanation could be that lactonization frequently occurs as a termination step in biosynthesis of hybrid peptides to perform cyclization with a PK-derived subunit (SIEBER & MARAHIEL, 2005). On the contrary, linear structures (32%) are prevalently peptides (23%). As a general observation we can also say that the majority of cytotoxic compounds is cyclic (68%).

The distribution of potency amongst the different structural classes as well as organisms of origin is neatly depicted in Fig. 1.11. It is clear that production of cytotoxic compounds is independent of genus of origin. On the other hand there are some noteworthy remarks to be concluded about chemotype-dependent potency. Most notably, polyketides can be classified as the least potent structures, followed by an alkaloids which possess much better potency, especially in the case of macrolide alkaloids demonstrating importance of biogenic ester bonds

and nitrogen moieties for the resulting activity. Altough the efficacy of peptides is relatively equally distributed, the hybrid entities are essential for highest activity strikingly proving that these structures are biosynthetically preffered with a reason and many SAR studies have been completed by nature in order to achieve such effectivity. Despite some connections have been deduced, the resulting potency probably depends more on an actual composition of cytotoxin, bestowing the molecule its unique conformational pattern required for interaction with targeted substrates, than simple chemotype. So, eventually, distribution of potency amongst peptides are rather random amongst genera.

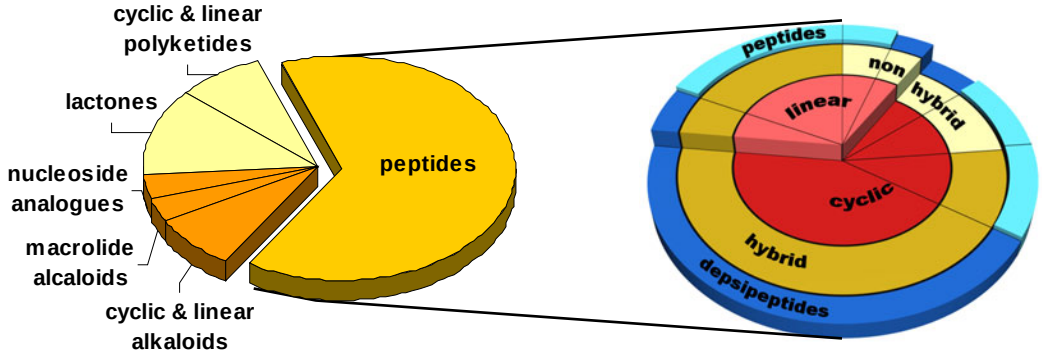


Fig. 1.10. Distribution of chemotypes amongst cyanobacterial cytotoxins

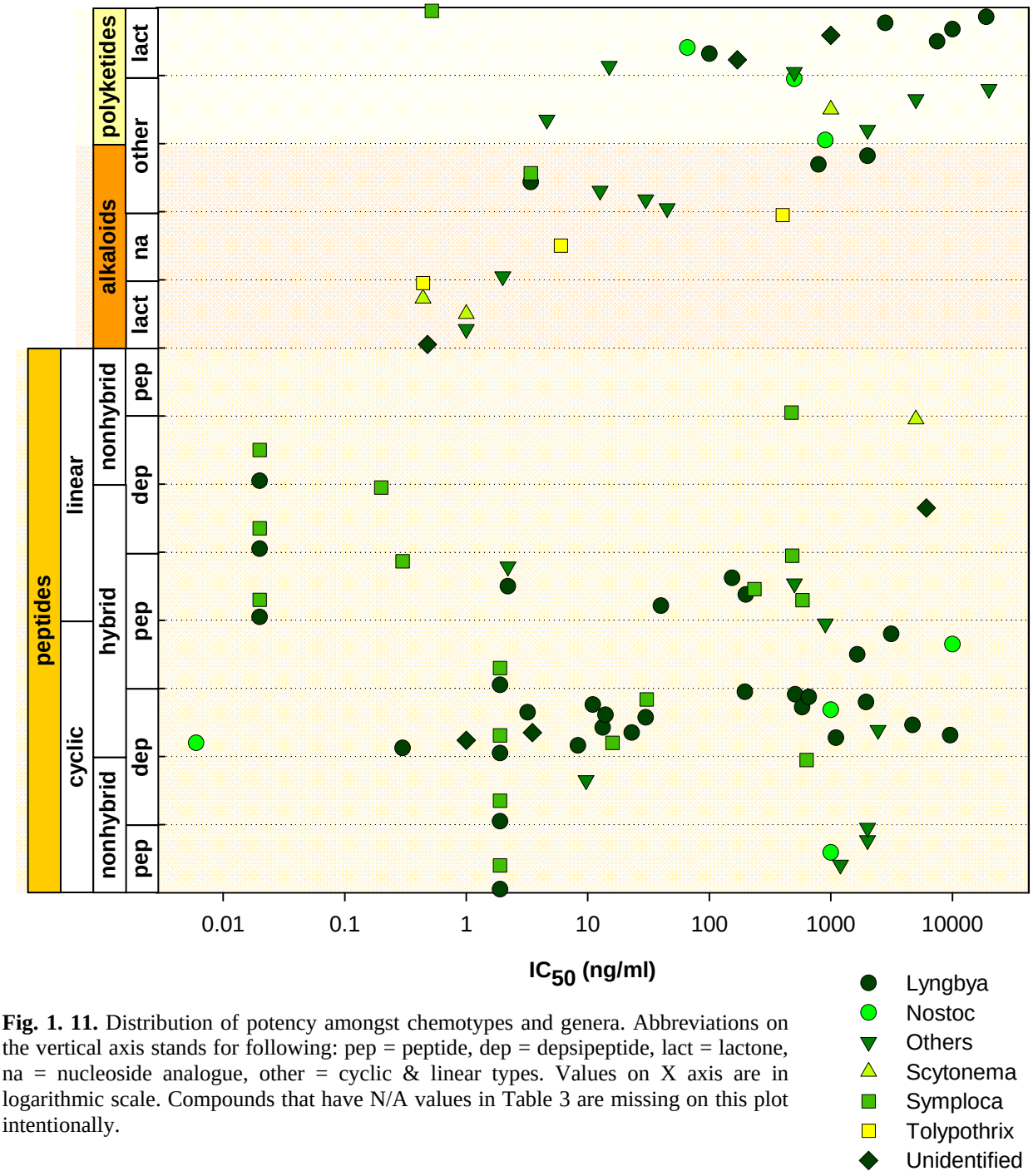


Fig. 1. 11. Distribution of potency amongst chemotypes and genera. Abbreviations on the vertical axis stands for following: pep = peptide, dep = depsipeptide, lact = lactone, na = nucleoside analogue, other = cyclic & linear types. Values on X axis are in logarithmic scale. Compounds that have N/A values in Table 3 are missing on this plot intentionally.

1.3.1.2. PHARMACOLOGICALLY ATTRACTIVE CYTOTOXINS...

1.3.1.2.1. ...in (pre)clinical trials

Currently only two of cyanobacterial cytotoxins are in pre/clinical trials (Curacins; Dolastatins 15,10) and the future of one is uncertain (Cryptophycins). All these compounds are microtubule disruptors, which is typical for natural products effective at pM – sub nM doses, but their potency is also what makes them even more toxic to organism than standard

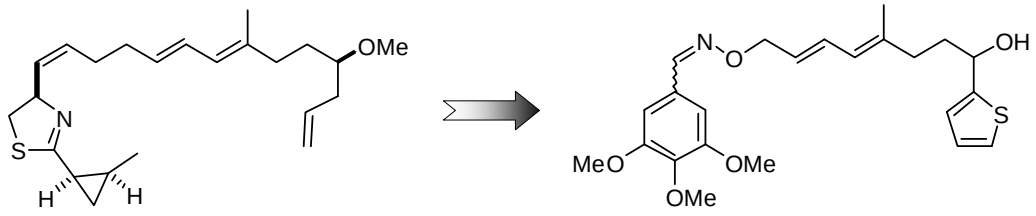
chemotherapeutics. But still, they are the most effective agents isolated from cyanobacteria so far with a lot of attention and invested efforts.

Curacin A

This unusual, relatively simple, lipidic heterocyclic alkaloid was first isolated from Curaçao strain of *Lyngbya majuscula* by Gerwick and colleagues in 1994 (GERWICK ET AL., 1994). No one expected that such a structure could be so potent antitumour agent (colon, renal and breast cancer-derived cells), and more importantly that it could inhibit microtubule assembly at nM concentrations (VERDIER-PINARD ET AL., 1998). Moreover, curacin A binds to β-tubulin subunit at colchicine binding site. If we now turn back to chapter 1.2.3.1 and compare the general structures of microtubule inhibitors binding there, it is clearly recognizable that such inhibitors require substituted aromatic cores. But in the case of curacin A, there is no similarity at all.

Unusual structural features (alkylated thiazoline, intriguingly distributed double bonds and stereochemistry) and unexpected biological activity led to rapid development of chemical synthesis of such relatively simple molecule (WIPF ET AL., 2002) and in the course of time three more congeners were isolated (B,C,D) all of them featuring only slight changes in the conformation not much affecting biological activity, but still, curacin A remained as the most potent (YOO & GERWICK, 1995; MARQUEZ ET AL., 1998).

Most crucial problems in clinical trials were the overall lipophilicity, oxidation-prone thiazoline and double bonds. But with its total synthesis elucidated, chemical analoguing was easily carried out. Altough there were many efforts to prepare agent with better pharmacological attributes, nearly all analogues that made the molecule clinically more suited also affected its activity (WIPF ET AL., 2002). It was found out that thiazoline ring and double bonds distributed across all molecule are both important for biological activity leaving only a little space for reasonable modifications. After many trials-and-errors one intriguing oxime based analogue emerged and is in preclinical testing. Altough the final hybrid molecule doesn´t resemble much its original, it retains its biological activity nearly perfectly, it is even more potent in inhibition of tubulin polymerization *in vitro* and its polarity much improved (WIPF ET AL., 2002).



Curacin A preclinical phase analogue

Dolastatins 10,15

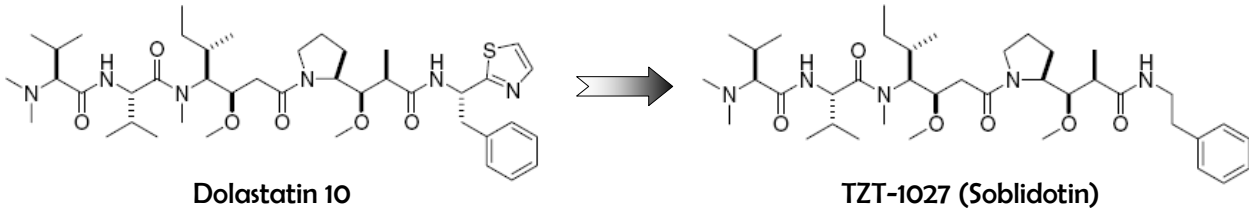
These are currently the most attractive and promising cyanobacterial metabolites in clinical trials. The first remarks about dolastatins dates back to 80. years of previous century when the first ones were isolated from sea hare *Dollabella auricularia* and recognized as extremely potent antiproliferative compounds (PETTIT ET AL., 1982B). Many more variants (~ 20),

including the most effective dolastatin 10 (linear peptide) and its counterpart dolastatin 15 (linear depsipeptide), were isolated in next decades also from cyanobacteria finally proving that this metabolite´s true source are cyahophytes *Lyngbya* and *Symploca* (HARRIGAN ET AL., 1998; LUESCH ET AL., 2001A).

Dolastatins 10,15 are microtubule assembly inhibitors active at nM – sub nM doses displaying tumour selectivity for melanoma, breast, colon, prostate and non-small cell lung cancers (NEWMAN & CRAGG, 2004). But as it is typical for such extremely active drugs, they display harsh side effects, like cardiomyopathy, neuropathy and poor host recovery behaviour, thus there is a need for optimized analogues, but as opposed to curacin, such structures are more challenging to produce synthetically (FLAHIVE & SRIRANGAM, 2005). There is no need to tell that these compounds are no exception regarding supply issue problem so common for many natural products, so synthetic chemistry is indispensable even for securing the steady source. Apparently, organisms don´t need surpluses of such effective chemicals in their biological warfare, thus the content/weight ratio is minimal, in most cases not enough to carry out even standard experiments, let alone clinical testing (FLAHIVE & SRIRANGAM, 2005).

Dolastatin 10 is linear pentapeptide composed of two very unusual γ-amino-β-methoxy acids (dolaproine and dolaisoleucine) with 3 asymmetric centers each and C-terminal thiazole-containing unit derived from phenylalanine (LUESCH ET AL., 2001A). Molecule contains totally 9 asymmetric centers, which render it very challenging for chemical synthesis. Due to exceptional activity, many efforts were invested in its preparation and results were successful allowing to synthesize analogues better suited for clinical application (SHIOIRI ET AL., 1993).

Dolastatin 15, on the other hand, is linear depsiheptapeptide, but resembling dolastatin 10 in many structural features like e.g. N-terminal N,N-dimethylvaline. Its own novelty is unique dollapyrolidone unit at C-terminus derived from N-acyl phenylalanine (PETTIT ET AL., 1989). Even in the case of dolastatin 15, the total synthesis was elucidated.

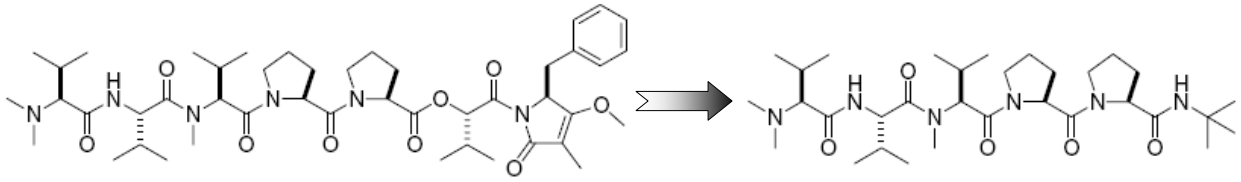


Dolastatin 10 TZT-1027 (Soblidotin)

Important thing to notice is that even if dolastatin 15 is more than 10x times less effective in pure tubulin assembly inhibition then dolastatin 10, it possess about 10x times higher activity towards inhibition of murine leukemia cell growth (L1210). This indicates more than one mechanism of action in play (FLAHIVE & SRIRANGAM, 2005).

SAR studies proved that „left“ (N-terminal) part of the molecule as well as absolute stereochemistry is vitally important for its biological activity, thus many synthetic analogues were prepared mainly substituting „right“ (C-terminal) part of molecule (FLAHIVE & SRIRANGAM, 2005).

One analogue of dolastatin 10 (soblidotin) is in phase I clinical trials displaying far more superior activity to standard tubulin disruptors like vinblastine or paclitaxel. Additionally, it induces tumour vasculature collapse making it exceptionally promising agent. Synthadotin (dolastatin 15 analogue) is an orally active third generation analogue recently promoted to phase II clinical trials (TAN, 2007). All information so far suggests these drugs will play an important role in future of chemotherapy.



Dolastatin 15 ILX-651 (Synthadotin)

Cryptophycin 1

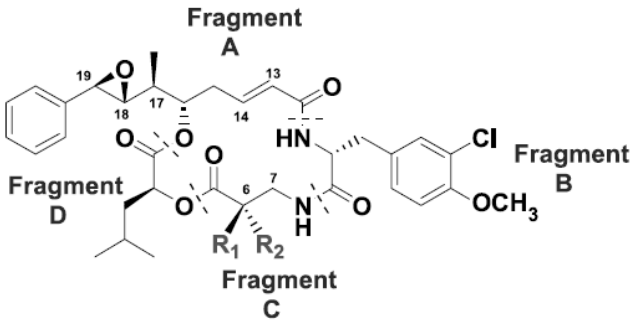
This compound is only cyanobacterial cytotoxin of terrestrial origin that made it into clinical trials, but that fact in no way discommends its powerful qualities, rather oppositely. Cryptophycin bears the prize of being the most potent microtubule disrupter ever isolated from natural sources (IC_{50} in units of pM) making it unmatched lead for anticancer application and even general scientific research. But unfortunately this extraordinary potency makes it a lot harder to tailor it for reasonable clinical application.

This toxin was first isolated in 1990 from terrestrial symbiotic *Nostoc* sp. strain ATCC53789 by Merck (SCHWARTZ ET AL., 1990) and secondly reported by Moore and coworkers in 1994 from *Nostoc* sp. strain GSV224 (TRIMURTULU ET AL., 1994). Paradoxically, its bioactivity was initially described as mere antifungal agent. Only later it was discovered that cryptophycin is potent cytotoxin irreversibly binding to tubulin with ability to destroy even drug-resistant cells (AL-AWAR ET AL., 2003). Total >25 variants was identified from nature so far, but cryptophycin 1 remained the most effective (ROHR, 2006). It showed outstanding antitumour activity both *in vivo* and *in vitro* against many taxol and vinblastine resistant cancers, including colon, mammary, pancreatic ductal adenocarcinomas, ovarian or non-small cell lung cancer (GOLAKOTI ET AL., 1996). Naturally, such properties made it the top priority in anticancer development.

Structurally, cryptophycin is intriguing macrocyclic depsipeptide displaying many unique traits. Molecule consists of four fragments (A,B,C,D) reflecting its biosynthetic subunits. There is no standard subunit in the structure. Fragment C contains β-amino acid alanine and in fragment B we can find both halogenated and methylated tyrosine. The rest is composed of two hydroxy acids (fragment A and D) fused to the molecule via amide and ester linkages. Fragment A is most interesting on the molecule because it contains an unusual PKS derived subunit with β-epoxide group, it is also the most challenging fragment to synthesize due to its four contiguous asymmetric centers (GOLAKOTI ET AL., 1996).

Unfortunately, cryptophycin possesses several adverse drawbacks which are mainly related to generally nonpolar character of compound (it requires excipient in order to be

intravenously administered), myalgia and neuropathy in treated patients, and rather decreased *in vivo* efficacy due to unstable moieties (AL-AWAR ET AL., 2003). Thus, the cryptophycin became the target for many synthetic groups.



Cryptophycin 1 (R_1 = H , R_2 = Me)
Cryptophycin 52 (R_1 = R_2 = Me)

Soon after its discovery, Barrow and colleagues put together first synthesis by convergent assembly of individual fragments (BARROW ET AL., 1995). Many SAR semisynthetic studies followed resulting in analogue cryptophycin52 which entered phase II clinical trials, where its journey to clinical application unfortunately ended in 2002 due to highly toxic side effects (ROHR, 2006). This analogue was probably chosen recklessly, because preclinical studies showed that there were better alternatives, but no large-scale biotechnological method to produce crypthophycins existed to achieve such a goal. So, with a lot of money wasted, the development of cryptophycin52 and other analogues have been shut down (ROHR, 2006). Noone wanted to explicitly revive this affair, but there were still many novel analogues emerging mainly focusing on modifying fragment A (AL-AWAR ET AL., 2004). SAR studies revealed that benzylic β epoxide moiety of fragment A, halogen presence in fragment B and all pendant methyls end their amount including fragment D are crucial for biological activity (AL-AWAR ET AL., 2003).

Latest discoveries seem to slowly reverse the sealed fate of cryptophycins. Magarvey and colleagues marked the complete gene cluster for cryptophycin biosynthesis and identified P450 oxygenase enzyme responsible for β epoxidation allowing cryptopphycin to be biotechnolocally produced via chemoenzymatic synthesis (MAGARVEY ET AL., 2006). Furthermore, they conducted successful experiments with precursor-directed biosynthesis, exploiting substrate tolerance of NRPS/PKS enzymes. Incorporating various starting units, aritifical amino acids and different halogens, they were able to obtain many novel analogues that were „nature-approved“, thus more interesting for clinical application. Maybe there is still a hope for the cryptophycin-based drug to emerge in a market some day.

1.3.1.2.2. ...as a promising novel leads

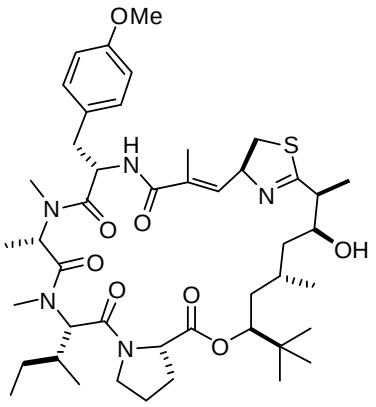
Despite the microtubule disruptors are extremely potent cytotoxic compounds prevalent among most active cytotoxins, they are clinically far from superior, thus there is an increased interest in finding natural leads with novel mechanisms of action or outstanding antitumor activities that could be the real rennaisance in chemotherapeutic treatment. It is rather controversial, why only microtubule interacting agents got into clinical trials, because actin is also targeted by many potent cyanobacterial products (e.g. tolytoxin, aplyronines), but unfortunately they never demonstrated reasonable activity *in vivo* (NEWMAN & CRAGG, 2004).

Indeed, cyanobacteria still hold many aces in their cells and the recent discoveries are proofs that their peculiar metabolic pathways are not even at the brink of depletion. Many of structurally fantastic compounds have been isolated recently featuring uknown MOA, interesting mechanistic novelty and superior antitumour activites, which are hands down feats worthy of promising leads.

I will briefly discuss the recent important structures in this field., intentionally omitting metabolites interacting with microtubules.

Apratoxin A

This cyclic depsipeptide, isolated from tropical specimen of *Lyngbya majuscula*, possesses one of the highest activities amongst the cyanobacterial cytotoxins. KB and LoVo cell lines growth were inhibited already at 360 and 520 pM, respectively. But unfortunately it demonstrated only marginal *in vivo* activity and no significant tumour selectivity. So far, four additional structural variants were isolated (B-E), all being inferior to the parent compound (LUESCH ET AL., 2001C).



Apratoxin A

Structurally, apratoxin A is of a hybrid PKS/NRPS origin displaying unusual O-methylated tyrosine and unique fatty acid moiety, 3,7-dihydroxy-2,5,8,8-tetramethylnonanoic acid (Dtena) (LUESCH ET AL., 2001C).

The compound induces G1-phase cell-cycle arest resulting in apoptosis, but exact mechanism of action couldn´t be attributed to any known molecular target at the time. Only recently it was found out, that apratoxin A-mediated cell death is performed by intriguing set of complex mechanisms very rare for natural products. It involves perturbation of JAK/STAT (Janus Kinase/Signal Transducers and Activators of Transcription) and cancer-related tyrosine kinase receptors (EGFR – Epidemal Growth Factor Receptor) signalling pathways dysregulated in many tumours (LIU ET AL., 2009).

In the case of JAK/STAT pathway, signalling cascade is interrupted by down-regulation of interleukine-6 signal transducer (gp130) crucial for Janus kinase, impairing its ability to transduce signal to effector STATs. STAT proteins are important transcription factors regulating cell survival, growth and differentiation and its dysregulation allow cancer cells to acquire many tumorigenic traits like e.g. immunosuppresion, enhanced angiogenesis or increased survival rate (SHEN ET AL., 2008).

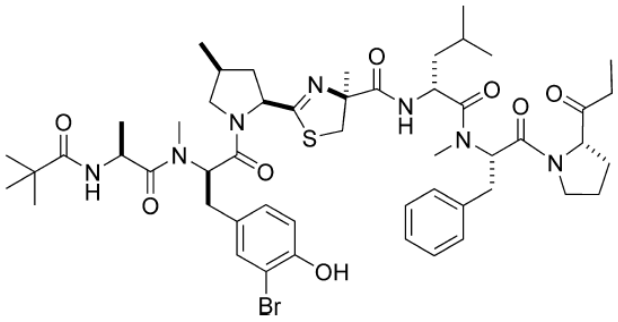
Deprivation of growth factors via EGFR tyrosine kinase pathway is not induced by direct intervention in signalling cascade as would seem most logical, but in a very ingenious way for a natural product. Apratoxin A inhibits cotranslational translocation of the EGFR and its proper folding in Hsp90 by mediating its breakdown through CMA (Chaperone-Mediated Autophagy) instead of typical proteasomal degradation. Perturbing EGFR secretory pathway is indeed

peculiar mode of action ultimately rendering the cell unable to accept extrinsic stimuli vital for its survival, proliferation and growth (SHEN ET AL., 2008).

This is extraordinary combination of MOAs with brilliant therapeutic value for drug development. The total synthesis of apratoxin was already devised and SAR studies are in progress (CHEN & FORSYTH, 2003).

Bisebromoamide

Another unique product, this time linear hybrid peptide isolated from japanese specimen of marine *Lyngbya* sp. Structure consists of seven units (only one being standard AA leucine) showing a lot of unique features in one molecule. Most notable attraction is N-methylated-bromo-tyrosine amino acid and two probably PKS derived units at both termini – 2-(1-oxopropyl)pyrrolidine (Opp) and pivalic acid (TERUYA ET AL., 2009A).

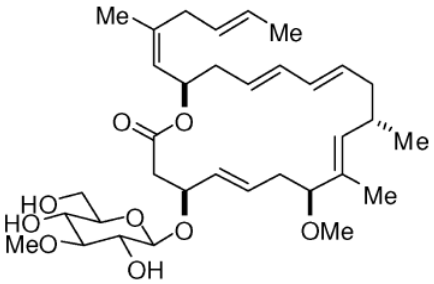


Bisebromoamide

Bisebromoamide inhibits HeLa S3 cell line with high potency (IC_{50} = 40ng/ml), but displays no significant antitumour activity. The slight glitch is balanced by rather interesting mode of action. This compound specifically inhibits phosphorylation of ERK, the effector kinase in complex signalling cascade important for proliferation, mediated by PDGF. This signalling pathway is overactivated in many cancer cells making it attractive molecular target in treatment (TERUYA ET AL., 2009A).

Biselyngbyaside

This relatively simple monoglycosylated macrolactone was isolated from the same location and organism as previous compound. The toxin exhibited IC_{50} 100 ng/ml towards HeLa S3 cell line and most importantly it showed attractive antitumour activity against CNS and lung cancers with GI_{50} ~ 50nM. Mechanism of action is unknown, but due to the COMPARE negative result, biselyngbyaside most likely features mechanistic novelty making it very promising structure (TERUYA ET AL., 2009B).



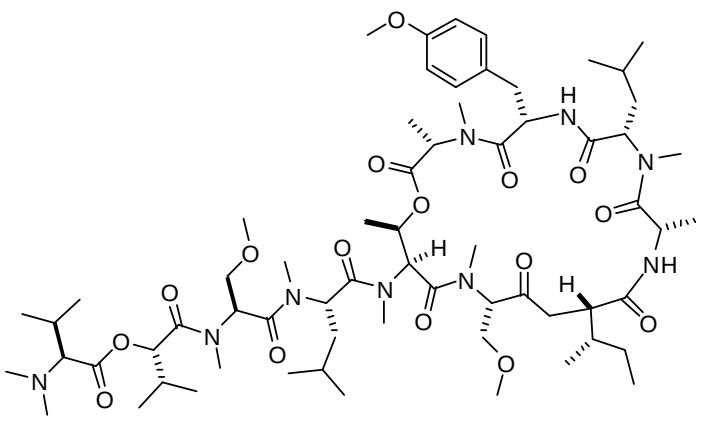
Biselyngbyaside

Coibamide A

This metabolite was isolated from *Leptolyngbya* sp. living in pristine waters of Coiba national park in Panama, protected by UNESCO. The structure of this cyclic depsipeptide displays unusually high ratio of N- and O- methylations with only one standard nonmodified amino acid alanine of all the 11 units. Such methylation pattern seems to be favourite motif in

cyanobacterial marine products probably bestowing them much needed resistance towards proteolytic degradation, unique properties and enhancing their potency and bioavalability profile. The presence of N,N-dimethylvaline and N,O-dimethylserine amino acids markedly displays this structural attribute exclusive for nonribosomal biosynthesis (MEDINA ET AL., 2008).

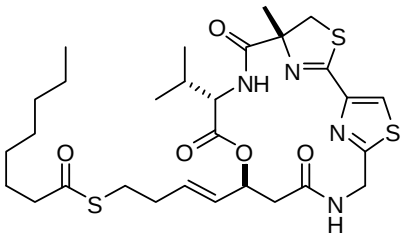
Coibamide A exhibited very potent antitumour activity with mean GI_{50} value 9.12 nM displaying good selectivity for CNS and breast-derived cancer cells. The molecule was COMPARE (PAULL ET AL., 1989) negative, thus it doesn´t affect any known molecular target and probably possess novel mechanism of action. It was found out that it induced accumulation of cells in G1 phase and their decrease in S phase (MEDINA ET AL., 2008) suggesting mechanism of action will revolve around DNA replication and signalling pathways. Its mechanistic novelty as well as perfect antitumour activity place Coibamide A on the top in the list of promising anticancer compounds of future.



Coibamide A

Largazole

Among all the recently discovered compounds, this one contains the most remarkable structural features. It is a cyclic hybrid depsipeptide which contains a thioster moiety never encountered in cyanobacteria before and 3-hydroxy-7-mercaptohept-4-enoic acid identified for the first time in natural product. Largazole was isolated from Florida Keys as a constituent of *Symploca* sp. (TAORI ET AL., 2008).

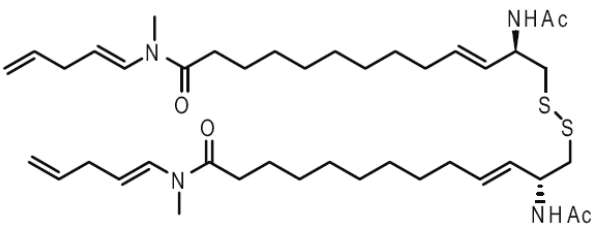


Largazole

Compound showed perfect selectivity for transformed cancer cell lines which is unprecendented in any other validated antitumour agents including doxorubicin and actinomycin.. Unfortunately its mode of action wasn´t elucidated yet (TAORI ET AL., 2008).

Somocystinamide A

Another structurally and bioactively unique object is this disulfide dimer composed of two identical linear lipopeptides. It was isolated from *Lyngbya majuscula /Schizothrix* sp. assemblage collected on Fiji (NOGLE & GERWICK, 2002).



Somocystinamide A

Somocystinamide A induces cell death

by caspase-8 mediated extrinsic apoptotic pathway by partitioning into plasma membrane and altering lipid compartments distribution. It has even ability to destroy cells resistant to DR-mediated cell death induced by standard agonists. Furthermore, somocystinamide A demonstrated excellent *in vitro* and *in vivo* activity against endothelial angiogenic cells, which are known for their susceptibility to this type of cell death (WRASIDLO ET AL., 2008). Altough it seems like caspase-8 will be bad therapeutic target defgenerated in many tumours, opposite is truth, and indeed many cancer cells maintain production of caspase-8, because it has important additional role in cellular motility and invasiveness (HELFER ET AL., 2006).

Apart from its rare MOA and antiangiogenic activity, it has another two favourable attributes. Firstly, unique, but structurally very simple molecule is far better platform for chemical synthesis than other metabolites of comparable activity, rendering the secured supply relatively smooth process. Secondly, its lipophilic nature makes it suitable for targeted delivery in nanoparticles (liposomes) and experiments proved, such incorporation has no effect on its bioactivity (WRASIDLO ET AL., 2008).

1.4. AIMS OF THE PROJECT

This master project represents a continuation of my previous work where I have been screening 65 cyanobacterial strains isolated from different habitats for cytotoxic activity against mammalian cancer cell lines. The research led to discovery of several cytotoxic strains notably of soil and symbiotic origin. After routine analysis of these cytotoxic strains on HPLC-MS it was found out that most of the compounds present in the MS spectra were unknown. Such a result offered large opportunity for discovery of novel cytotoxic metabolites.

Thus, one of the active compounds, present in soil cyanobacterium *Nostoc muscorum* Lukešová 14/86, has been selected for thorough investigation of its unknown cytotoxic structure and elucidation of mechanism of action responsible for death-inducing effect in cancer cell lines. The main tasks were following:

Analytical chemistry:

- Designing of the most efficient large-scale extraction procedure with respect to further HPLC separation
- Purifying the cytotoxin
- Characterizing the molecule and describe its 2D structure

Cell toxiclogy and mechanism of action:

- Determining the IC_{50} value (potency) and comapring it to other metabolites
- Elucidating the mechanism of action of cytotoxin
- Evaluating potential pharmacological or biotechnological application

2. EXPERIMENTAL PROCEDURES

2.1. ANALYTIC CHEMISTRY

2.1.1. CULTIVATION AND EXTRACTION OF BIOMASS

Large-scale cultivation of *Nostoc muscorum* Lukešová 14/86 strain, isolated from arable field (Nezamyslice, Czech Republic) in 1991, proceeded in custom-made 150 l glass cuvette. It was grown in A-D Anabaena medium (ARNON ET AL., 1974), bubbled with CO_2 enriched air (2%) at constant temperature 28°C for 10 days. Such period was proven to be the best for this strain to obtain maximum yield of toxin/biomass ratio (data not shown). The cultivation was performed during still relatively sunny October 2008 in a well-ventilated glasshouse, so during day there was no need to use artificial light and at night the cyanobacteria were irradiated by lamp tube panel set to deliver 70W/m^2 light intensity. After 10 days passed, culture was harvested by simply draining the cuvette into collecting vessels, left to sediment for appx. 15 mins and, because the toxin is preserved mainly in cells, medium was carefully discarded to obtain the biomass. Biomass was transferred on to the plastic dishes and stored at -80 °C and as a final step, freeze-dried to provide completely dry matter.

On the following lines I will describe the complete extraction protocol. But as it is result of my own development process, it will be also dicussed later on in results.

2.1.2. LARGE-SCALE EXTRACTION (SCREENING FOR OPTIMAL EXTRACTION SOLVENT)

Every extraction batch was performed manually homogenizing 6 g of biomass with appx. 300 ml of 50 % MeOH, transferring the product into 100ml pyrex tubes and kept in the dark for 1 hour. Then, the extract was centrifuged at 3750 RCF for 15 minutes, resulting supernatant poured into glass cylinder and biomass re-extracted again following same procedure. Biomass was re-extracted once more following the same protocol. Finally, the raw extract was diluted to concentration 10 mg/ml (biomass/solvent) and kept at -4 °C overnight.

Screening for extraction solvent was done in a similar fashion, but at a smaller scale. 200 mg of biomass was homogenized with appx. 10 ml of selected solvent, the product transferred into glass tube and kept in the dark for 1 hour. Then, the extract was centrifuged at 3750 RCF for 15 minutes, resulting supernatant was evaporated using rotary vacuum evaporator (37°C) and resuspended in 1 ml of 70 % MeOH in case of every extraction solvent. Biomass was re-extracted twice more following the same protocol. Prior to HPLC-MS analysis, resulting extracts were centrifuged in minicentrifuge at 12200 RCF for 10 minutes and filtered through 0.45 μm filter to remove any undissolved particles and solid substances.Extracts were then subjected to standard HPLC-UV/DAD-MS analysis developed for this strain (Chapter 2.1.6) and effectivity

of every solvent was evaluated according to the integral of EIC of both molecular ion and sodium adduct of cytotoxin. DAD at 440 nm spectra was also evaluated as a secondary discrimination factor regarding the pigment content. Following set of solvents was used in the screening: hexane, toluene, dichloromethane, chloroform, *n*-butanol, tetrahydrofuran, *n*-propanol, ethylacetate, dioxane, ethanol, acetone, acetonitrile, methanol (50 %, 70 %, 100 %), acetic acid (5 %, 20 %), water. Every extraction experiment was performed in at least three repeats.

2.1.3. EXTRACT PARTITIONING (DETERMINING THE BEST RATIO AND SOLVENT)

Extract was partitioned with EtOAc (ethylacetate) and water in the ratio 2:1:3 (extract:EtOAc:distilled water) in 2 l glass funnel totalling 1800 ml. Individual constituents were added in the following order: extract, EtOAc and water. After every addition, the contents were mixed by swiftly turning the vessel upside down 3x times. Mixture was left to equilibrate in a ventilated cabinet to the next day. Water phase was then drained and EtOAc phase subjected to second partitioning by equal volume of water. After appx. 2 hours, water phase was drained again and combined with previous partition. Such prepurified extract was evaporated roughly for 30 min at 37 °C to remove the residual EtOAc. For given task, more than adequate indicator of successful evaporation was simple olfactory sense.

In the experiments dealing with the partitioning optimization, extract was first combined with selected solvent in the 50 ml glass funnel (ethylacetate, dichloromethane, butanol or toluene) at various ratio, 3x times mixed by turning the vessel upside down and then distilled water was slowly added and mixed again in same manner. Ratio of participating solvents was tried to keep as minimal as possible and first visible and stable phase separation was considered as a discrimination factor for the minimal volume of water that needs to be added. After partitioning, the water/extract phase was drained or aspired by pipette, depending on the density of nonpolar solvent, evaporated (37 °C – 42 °C) and concentrated as the original extract. It was then analysed on HPLC-UV/DAD-MS and evaluated according to the same protocol as described in previous section. Additionally, measurements of UV/VIS spectra of prepurified extracts were carried out on double-wavelength/double beam spectrophotometer (Shimadzu UV-3000) spectrometer. Factors influencing the result were effectivity at removing of ballast substances, retention of active compound, feasibility, ease of execution and finally also the volume of chemicals used.

2.1.4. CONCENTRATING OF EXTRACT

Prior to preparative HPLC separation of active compound, the partitioned extract was concentrated using SPE (Solid Phase Extraction) method. SPE cell with 12-socket lid (Supelco Visiprep, Sigma) was equipped with 4x Oasis HLB cartridges (Waters, Prague) and, according to manufacturers protocol, the prepurified extract was run through them at a lowered pressure.

Adsorbed compounds was then eluted from the sorbent by 100% MeOH in 3x1 ml steps. Resulting vextract gathered from all cartridges were transferred into heart-shaped flask and evaporated at 37°C, resuspended in a mixture of solvents consistent with initial gradient of preparative HPLC at 1.2 g/ml (biomass/solvent), centrifuged and filtered through 0.45 μm filter. To check the efficiency of SPE, extract was analysed on HPLC-UV/DAD-MS before and after treatment.

2.1.5. PREPARATIVE HPLC PURIFICATION

Preparative separation and fractionation of active compound was carried out on LabAlliance HPLC system (Watrex). Spectra were monitored at 220 nm and column chamber heated to 30 °C. Eventually, two successive chromatographic steps were developed to obtain the pure product. Before each fractionation step columns were thoroughly washed by the strongest composition of mobile phase for 30 minutes at lower flow rate and equilibrated by starting point mobile phase for 15 minutes. Mobile phases containing MeCN were manually premixed, sonificated and heated (15 min, 30°C) prior to any HPLC measurement.

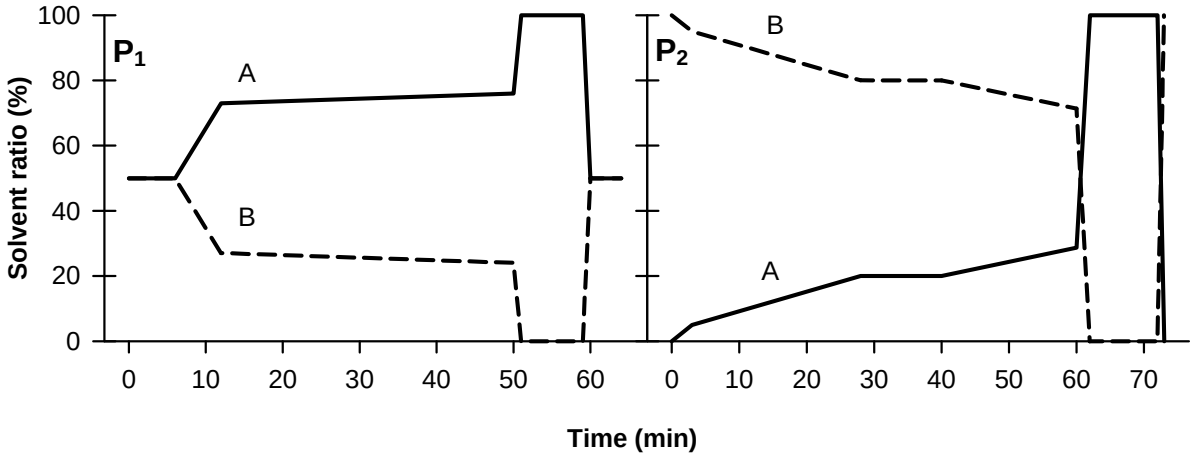


Figure 2.1. Preparative HPLC gradients P_1 and P_2. P1 (A = MeOH, B = H_2O). P2 (A = 90% MeCN:MeOH – 9:1, B = 30% MeCN:MeOH – 9:1).

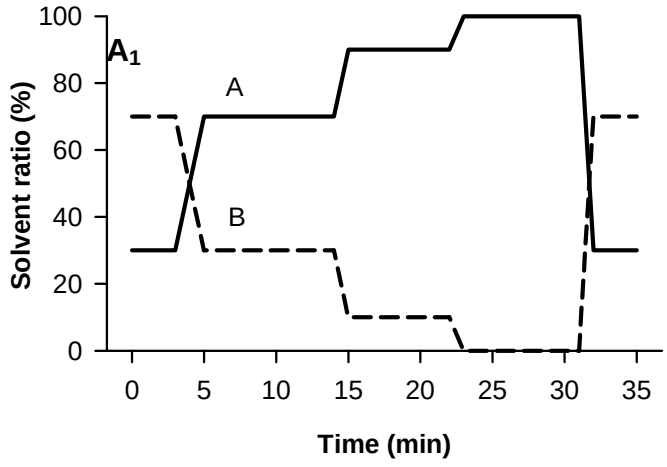


Figure 2.2. Analytic HPLC gradient A_1. A = MeOH, B = H_2O

In the first one, initial extract was separated on C18 reversed-phase column (Reprosil 100, 250x10 mm, 5μm, Dr.Maisch GmbH) eluted by $MeOH^{A}/H_2O^{B}$ gradient P_1 (flow rate = 2.6 ml/min, injection = 0.5 ml) (Fig. 2.1). Cytotoxic fraction was collected in to a glass vessel wrapped in tinfoil and cooled on ice. After whole extract was chromatographed, eluate was evaporated and resuspended in MeCN:MeOH:H_2O (27:3:70) at final

concentration 4 g/ml (biomass/solvent) and subjected to second phase separation. This time, separation run on a larger C18 reverse-phase column (Reprosil 100, 250x25mm, 5μm, Dr.Maisch GmbH) eluted by 90% (MeCN:MeOH - 9:1)^{A}/30% (MeCN:MeOH - 9:1)^{B} gradient P_2 (flow rate = 17 ml/min, injection = 1 ml) (Fig. 2.1). Collection of fractions was done in the same manner as in previous separation step. Purified cytotoxin was then evaporated using rotary vacuum evaporator and dessicated under nitrogen stream. Final drying was performed in vacuum exsiccator filled with silicagel.

2.1.6. HPLC-UV/DAD-MS ANALYSES

Analytical-scale chromatographic measurements were executed on an Agilent HP 1100 HPLC-MS system equipped with ESI ionization interface and HP 100 MSD SL-Ion trap operated by Chemstation software. Parameters of ionization and trap were at its default settings during all routine analyses. Separation proceeded on C8 reversed-phase column (Zorbax Eclipse-XDB, 150x4.6mm, 5μm, Agilent) eluted by MeOH^{A}/H_2O^{B} (+ 0.1% formic acid) gradient A_1 (flow rate = 0.6 ml/min, temperature = 30°C, injection = 20μl) (Fig. 2.2).

2.1.7. NMR ANALYSES

NMR spectra were measured using Bruker spectrometer AVANCE III 600 (observational frequency 600.23 MHz for 1H and 150.93 MHz for 13C) in CD3OD (ARMAR Chemicals, Döttingen, Swiss) at 30 oC. Residual signal of solvent served as an internal standard (δH 3.330, δC 47.30). 1H NMR spectrum was supplemented by twofold memory points and prior to Fourier transform multiplied by scaling function to enhance the resolution (negative exponential plus Gauss function. 13C NMR spectra were enhanced by artificial line widening (1 Hz) due to elevated signal to noise ratio. Assignment of signals was based on 2D NMR experiments – COSY (Correlation Spectroscopy), HSQC, HMBC (Heteronuclear Multiple Bond Correlation), NOESY (Nuclear Overhauser and Exchange Spectroscopy) and ROESY (Rotating Frame Overhauser Enhancement Spectroscopy) carried out on manufacturer supplied software (Bruker BioSpin GmbH, Rheinstetten, SRN).

2.1.8. HIRES MS ANALYSES

Stock solution of muscotoxins (10 μg/ml) for MS experiments were prepared in 50% MeOH with addition of 0.1% formic acid. Measurements were performed on a commercial APEX-Qe FTMS instrument equipped with a 9.4 T superconducting magnet and a Combi ESI/MALDI ion source (Bruker Daltonics, Billerica MA, U.S.A.) using electrospray ionization. The flow rate was 1 μl/min and the temperature of dry gas (nitrogen) was set to 200°C. The Q front-end consists of a quadrupole mass filter followed by a hexapole collision cell. By switching the potentials on the exit lenses appropriately under the control of the data acquisition computer, ions could be accumulated either in the hexapole of the Combi ESI source, or in the hexapole

collision cell of the Q front end, prior to transfer to the FTMS analyzer cell. Mass spectra were obtained by accumulating ions in the collision hexapole and running the quadrupole mass filter in non mass-selective (Rf-only) mode so that ions of a broad *m/z* range (150-2000) were passed to the FTMS analyzer cell.

The species of interest were isolated in the gas-phase by setting the Q mass filter to pass the *m/z* for ions of interest within a 3.0 *m/z* window. After a clean selection of the desired precursor, ion had been confirmed and fragmentation was induced by dropping the potential of the collision cell (12V). All MS and MS/MS spectra were acquired in the positive ion mode with the acquisition mass range 150-2000 *m/z* and 1M data points collected. It results in 200000 maximal resolution at 400 *m/z*. The accumulation time was set at 0.5 s (1.5 s for MS/MS), the cell was opened for 4500 μs, 8 experiments were collected for one spectrum. The instrument was internally calibrated using triply and double charged ions of angiotensin I, quintuple and quadruple charged ions of insulin. It results in typical mass accuracy below 1 ppm. After the analysis the spectra were apodized using sin apodization with one zero fill. The interpretation of mass spectra was done using DataAnalysis version 3.4 software package (Bruker Daltonics, Billerica MA).

2.1.9. CHIRAL AMINO ACIDS ANALYSIS

Amino acids were hydrolysed by 6 M HCl at 110°C for 24 h, and the derivatization with heptafluorobutyl chloroformate (SIMEK ET AL., 2008) was performed in order to reveal the absolute amino acid configuration. The chirality of the released amino acids (as the corresponding N(O,S)-heptafluorobutoxycarbonyl-heptafluorobutyl derivatives) were determined by gas chromatography, with a flame ionization detector on a 25 m x 0.25 mm ID x 0.12 μm Chirasil-L-Val column (Varian Inc., Palo Alto, CA, USA) using a method described elsewhere (ZAHRADNICKOVA ET AL., 2009).

2.2. CELL TOXICOLOGY AND MOA DETERMINATION

2.2.1. CELL CULTURES & HANDLING

Cancer cell lines Sp/2, YAC-1 (semi-adherent and non-adherent murine lymphoblastomas, respectively) and HeLa (adherent human cervical cancer cell line) were kindly provided by Assoc. Prof. Jan Kopecký from Institute of Parasitology of Czech Academy of Sciences. Mouse fibroblasts were supplied by Assoc. Prof. Jan Černý from Charles University in Prague.

YAC-1, Sp/2 and HeLa cells intended for use in IC_{50}, toxic effect dynamics, LDH or SYBR Green experiments were cultivated in RPMI-1640 medium w/o L-Gln (E15-039, PAA), supplemented with 5% (YAC-1, Sp/2) or 10% (HeLa) bovine foetal serum (A15-151, PAA), 300 μg/ml final concentration of freshly dissolved L-glutamine (Sigma), and 1% antibiotic-antimycotic solution (PAA, P11-002). Cells were grown in tissue culture flasks (NUNC) in CO_2

incubator at 37°C and high humidity. Fibroblasts and HeLa cells intended for microscopy were cultivated in DMEM medium w/o L-Gln and phenol red (Gibco, Invitrogen) with same supplements. Cells were maintained according to standard procedures, YAC-1 were subcultured simply by diluting with fresh medium, Sp/2 cells were blown off from the bottom of the flask by the pipette prior to subculturing, and HeLa and fibroblast cells were trypsinized by 1x Trypsin/EDTA solution (PAA, L11-003). In the beginning of every experiment, the condition of cells were inspected with regards to its viability (Trypan blue exclusion dying) (BERG ET AL., 1972) and morphology (inverted bright field microscope). Only cell cultures with a proper morphology and viability > 95% were used.

For all further experiments, the stock solution of purified cytotoxin was prepared in 100% DMSO at 4 mg/ml and kept at -20°C.

2.2.2. MTT CYTOTOXIC ASSAY

Assay was executed according to (MOSMANN, 1983) with slight alterations. Cell harvest of HeLa cells was centrifuged at 108 RCF in a cooled (4°C) centrifuge chamber for 15min (Sorvall Evolution RC, Kendro Laboratory Products). Supernatant was discarded and cell pellet resuspended and homogenized in 5-10 ml (according to expected number of cells) of fresh RPMI-1640 medium. Cells were counted in a Bürker´s chamber and concentration adjusted to $2.5x10^5$ cells/ml. 100 μl of obtained cell suspension was pipetted into inner wells of 96-well transparent microtiter plate (Nunclon delta surface, NUNC) avoiding the marginal ones, that were later filled with 250μl of deionized water to reduce excessive evaporation of inner wells. The plates were put into incubator for appx. 20 hours. This period (including cell-spreading) was proved in our laboratory conditions to be a doubling time for the HeLa cell line, so next day cells were well-spread at estimated final concentration $5x10^4$ cells per well. Same process was followed with YAC-1 and Sp/2 cells with exception of being processed right before the experiment and seeded at concentration $7.5x10^4$ cells per well.

Afterwards, cells were treated with freshly prepared concentration series of cytotoxin (diluted in cultivation medium) at a final concentration of 1-30 μg/ml in 1% carrier solvent. Reference cells were treated with a blank carrier solvent (1% DMSO). Each treatment was performed in triplicate and in 200 μl final volume per well.

The plates were put into incubator for 24 hours (37°C and 5% CO_2) and after the exposition time elapsed, MTT (3-(4´,5´ dimethylthiazol-2´-yl)-2,5-difenyltetrazolium bromid; Sigma-Aldrich) was added into each well at final concentration 100 μg/ml and plates were placed back into incubator for 4 hours. Prior to use, MTT was dissolved in sterile 1x PBS and filtered through a bacteriological filter (PES membrane 0.22 μm, Millex GP).

Microtiter plates were then centrifuged at 1980 RCF for 15min, supernatant shaken off and crystals redissolved in 200μl of DMSO. Absorbance values were measured on UV/VIS microplate reader (Sunrise, TECAN) at 590 and 640nm (measurement and reference wavelength, respectively). Before each measurement plates were shaken for 3 minutes at highest intensity.

The mean absorbance values of treated cells triplicates were set against the mean of reference triplicates and resulting ratio multiplied by 100 represented viability of treated cells in percentages. IC_{50} was determined by fitting the data with dose-response curve and regression analysis.

2.2.3. ALAMARBLUE ASSAY

Experiment was carried out following the same procedure as in previous section up to the point of adding the MTT. Instead, 3 hours before the end of exposition, Alamarblue solution (Serotech, Nalgene) was added into each well at 10% final concentration and absorbance recorded at 570 and 600 nm (measurement and reference wavelength, respectively) immediately and every hour up to the end of exposition (4 values). Measurement proceeded at 37°C and prior to each one, plates were gently shaken by the machine for 30 s. The absorbance values obtained from each reference well were plotted against time and curves were verified for presence of incremental tendency. Difference between the initial and end-point absorbance value was used in calculation of viability and subsequent IC_{50} determination as described in former section.

2.2.4. MONITORING THE DYNAMICS OF TOXIC EFFECT

HeLa cells were prepared as in MTT cytotoxic assay, but this time the final seed concentration was $1.25x10^4$ cells per well in 100 μl of fresh medium. Next day, 50 μl of medium was removed and cells were exposed to 50 μl concentration series of toxin or other compounds in triplicates at desired final concentration in 1% carrier solvent (typically DMSO). Reference control cells were treated with same final concentration of carrier solvent as exposed cells. Sterile Alamarblue was then added at 10% final concentration to every control and exposed well and additionally to one triplicate of wells containing no cells. After initial measurement with same parameters as in previous section the cells were put into incubator and absorbance values collected every hour during 24h exposition. Mean value of blank wells (no cells) was substracted from mean values of every triplicate and absorbances plotted either as ΔA/1h or unaltered values against time and fit by the suitable curve. Shape and parameters of the curves served as an evaluating factors. All graph-related activites were conducted in GraphPad Prism v5.0 (GraphPad software).

2.2.5. IMMUNOFLUORESCENCE MICROSCOPY AND TIMELAPSE IMAGING

All images were acquired using inverted fluorescence microscope (Olympus Cell^R system, microscope Olympus IX81) with homonymous dedicated software. HeLa and fibroblast cells were seeded on to 12mm round sterile coverslips mounted in 24-well microtiter plates (NUNC) at ~ 3 - $5x10^5$ cells per well in 1 ml. Next day, cells were treated with compounds in similar fashion as in section 2.2.2.

After the end of exposition cells were fixed in 3,8% formaldehyde for 20min, washed with PBS once, then the residual formaldehyde was removed by quick reaction with NH_4Cl for 5 minutes and after that, samples were thouroughly washed. In next step, cells were permeabilized by 0,5% Triton X-100 in PBS (w/v} for 1 minute and again washed with PBS. After washing, 2% BSA was used to block non-specific antigens for 10 minutes. Next, the cells were incubated with immunoflourescent probes or antibodies for one hour and consistently washed with 2% BSA. Coverslips were removed from plate and glued onto the microscopic slides with DAPI solution (1 μg/ml in 10% Mowiol). Slides were left overnight in refrigerator to solidify and evaluated next day on the fluorescent or confocal microscope.

All immunoflourescent conjugates were obtained from Molecular Probes, Invitrogen. F-actin was stained with Alexa Fluor® 488 phalloidin, tubulin with Alexa Fluor® 555, mitochondria with MitoTracker® Red CMXRos and receptor-mediated endocytosis was tracked by transferrin Alexa Fluor® 488 conjugate. Live tracking dyes (tranferrin, MitoTracker) were loaded to the wells 30 min before the end of exposition.

Timelapse observation proceeded in thermoregulated microscopic culltivation chamber (Pecon) (37°C, 5% CO_2). Fibroblast cells were grown on 25mm coverslips in 6-well microtiter plates. 30 minutes prior to start of experiment, the coverslip with cells were transferred into a cell cultivation system (Pecon) filled with 0.5 ml of DMEM medium and loaded with MitoTracker® dye. Then it was placed into the chamber and cells were left to adapt to the altered conditions for a while and finally exposed to a various concentration of cytotoxin. Frames were shot every 15 seconds and video created and edited in an image processing and analysing software (ImageJ, Opensource freeware). This software was also used for editation of still fluorescent and DIC images.

2.2.6. LDH LEAKAGE ASSAY

CytoTox 96™ Assay kit was bought from Promega (Eastport, CZ) and experiment executed according to the manufacturers instructions. HeLa cells were seeded at $1x10^4$ cells per well in 50-200μl of medium, depending on the length of exposition time, and exposed using standard protocol, but this time black plates were used instead of transparent ones to reduce the fluorescence cross-talk (GAMBLE & MURIANA, 2007). Each exposition time experiment was evaluated exclusively in one plate. Actual measurements were performed on microplate reader (Infinite 200, TECAN) equipped with Xfluor4 software and appropriate excitation and emmision slides (570 and 595 nm, respectively).

2.2.7. FLUORESCENCE MEASUREMENT OF CYTOSOLIC CA^{2+}

HeLa cells cultivated on modified glass coverslips (cut into rectangles to fit the sample holder in the spectrometer) in a manner described in section 2.2.1 were firstly washed in modified HBSS medium (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 3 mM $MgCl_2$, 10 mM Hepes-Na, 50 mM glucose (pH 7.4)). After washing, cells were loaded with 3 μM Fura-2

acetoxymethyl ester (Fura-2/AM) for 30 min at 25 °C in the dark, rinsed, and allowed to rest in HBSS for 30 min prior to fluorescence measurements of $[Ca^{2+}]i$ at 25 °C. Ratiometric rapid screening and multiply repeated measurements were performed using FluoroMax-3 spectrofluorometer equipped with DataMax software (Jobin Yvon Horriba, France). The observed area of coverslip mounted in the 1-cm cuvette was about 10 mm^2, corresponding to approximately 10^4 cells. Fluorescence intensity of Fura-2 (excitation wavelengths 340 and 380 nm, emission wavelength 510 nm) was recorded every 15 s, and integration time for each wavelength was 3 s. The measured fluorescence intensity was not corrected for background intensity (<10%).

2.2.8. ARTIFICIAL BILAYER PREPARATION

First of all, large unilamellar vesicles (LUVs) had to be prepared by mixing chloroform solutions of POPC (Palmitoyl-oleoyl-phosphatidylcholine) (molar 60 %), Cholesterol (molar 20 %), DOPE (Dioleoyl-phosphatydilethanolamine (molar 10%), Sphingomyelin (molar 10%) in a glass tube with selected fluorescent dye (2% final concentration in LUVs). Solvents were evaporated under a stream of nitrogen while being continuously heated to 50 ^oC. The dry lipid film was then suspended in a 50 mM Tris-HCl buffer (150 mM NaCl, pH 7.5) and vortexed for 4 min. LUVs were formed by extrusion through a polycarbonate membrane (Avestin, Ottawa, Canada) with 100 nm pores. The prepared samples were transferred to a 0.3 cm quartz cuvette and equilibrated at the desired temperature for 10 min before each measurement. The final concentration of phospholipids in the cuvette was 0.3 mM.

2.2.9. STEADY-STATE FLUORESCENCE MEASUREMENT OF LUVS

Steady-state excitation and emission spectra were recorded on Fluorolog-3 spectrofluorometer (model FL3-11; Jobin Yvon Horriba, USA) equipped with a Xenon-arc lamp at 25 °C, and at 37 °C. All spectra were collected in 1 nm steps (2 nm bandwidths were chosen for both the excitation and emission monochromators). The temperature in the cuvette holder was maintained within ±0.1°C using a water-circulating bath. When recording emission spectra of Patman and Laurdan, the samples were excited at 370 nm. The steady-state anisotropy of TMA-DPH was measured with polarizers inserted into the set-up at the excitation wavelength of 350 nm and emission wavelength 450 nm with 4 nm bandwidth. Four intensities of polarized fluorescence (I_{VV}, I_{VH}, I_{HV}, I_{HH}) were recorded, where "V" and "H" in the subscripts represent the vertical and horizontal directions, and the first and second subscript denotes the direction plane of the polarization in the excitation and emission arm, respectively. The steady-state anisotropy is calculated as follows:

$$r_{st} = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

in which G is the instrumental correction factor given by the observed ratio, I_{HV}/I_{HH}.

2.2.10. CARBOXYFLUORESCEIN ASSAY

The wavelengths of machine were set at 492 nm and 518 nm for excitation and emission, respectively. The LUVs containing 8 mM carboxyfluorescein were stirred continuously. After the addition of compounds, time evolution of steady-state intensity was observed till it became constant. Then, Triton X-100 (1% final concentration) was added to determine 100% reference point of carboxyfluorescein leakage.

2.2.11. CHEMICALS

All solvents for extraction and HPLC analyses were supplied either by Sigma-Aldrich (Germany) or Analytika (Czech Republic) and were of HPLC-grade purity. Microcystin-LR was purchased from Cayman Chemicals (USA), Taxol and Cyclosporin A was a gift from TEVA Pharmaceuticals (Czech republic).

All lipid constituents were purchased from Avanti lipids (USA), Laurdan, Patman and TMA-DPH were purchased from Molecular Probes (Eugene, OR), Carboxyfluorescein and the chemicals needed for the buffer preparation were purchased from Sigma-Aldrich (Germany).

3. RESULTS

3.1. ANALYTIC CHEMISTRY

Based on the presence of quasimolecular ions found in mass spectra of TIC (Total Ion Current) chromatogram of cyanobacterium *Nostoc muscorum* Lukešová 14/86 strain, following five principal compounds were identified (Fig. 3.1): ***m/z* 886** (Rt 10.5 min.), ***m/z* 900** (Rt 11 min.), ***m/z* 1098** (Rt 12.5 min), ***m/z* 1112** (Rt 13.5 & 15.1 min) and ***m/z* 1212** (Rt 18.5 & 20.7 min). Structures with ***m/z* 885** and **1111** were tentatively identified based on their molecular weights as already known cyanobacterial metabolites microginin 299-A and oscillapeptin G, respectively. Other observed compounds were ascertained to be unknown.

Due to the significant cytotoxic activity and presence of unknown compounds, the extract was subjected to bioactivity-guided fractionation. Fraction containing metabolite with ***m/z* 1212** was found to be exclusive active component. Because no congruence of this mass with known compounds in literature was found, the novel cytotoxin was selected for detailed study. In the rest of the text this cytotoxic compound will be reffered to as **muscotoxin or cytotoxin**.

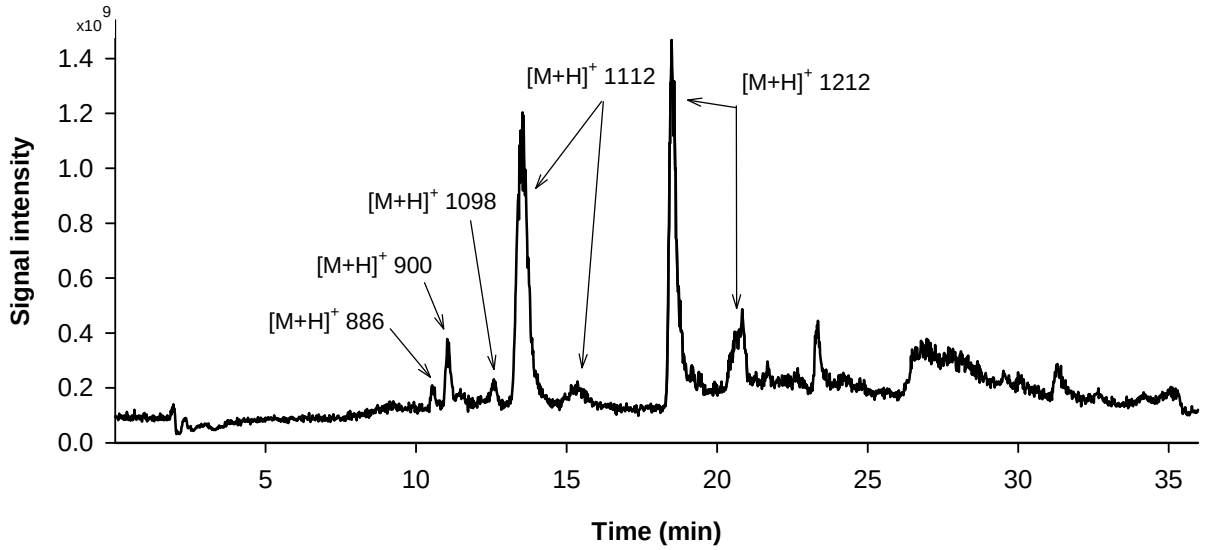


Figure 3.1. HPLC-MS chromatogram (TIC) of methanolic extract of *Nostoc muscorum* strain. The most intensive quasimolecular ions for given Rt are shown. The compounds eluting from Rt 23 min to the end of analysis were identified in our laboratory to be contaminants and pigments.

At first, there was a need to develop maximally efficient extraction and purification protocol to obtain enough pure product for structural, toxicological and MOA (Mechanism Of Action) experiments.

As a first step in this procedure it was a selection of the most suitable extraction solvent. In the screening of extraction efficiency of 19 organic solvents (Fig. 3.2) it was found out that 100% MeOH (methanol) was the most effective extraction solvent followed by aqueous MeOH in 70% – 50% solutions. MeOH in 70% solution extracted only 2.6% lower amount of the

muscotoxin, 60% MeOH solution 4%, and 50% MeOH solution 10.4% less of muscotoxin in comparison to pure MeOH. There was an abrupt change in effectivity of 30% MeOH showing extraction efficiency only 58% which made it merely 2% more efficient than extraction with pure distilled water. Enrichment of water by 5% acetic acid didn´t exhibit any significant improvement. Series of alcohols displayed unusual disparities. EtOH (ethanol) was still fairly efficient (85% of pure MeOH extraction power), but *n*-PrOH (*n*-propanol) and *n*-BuOH (*n*-butanol) on the other hand displayed only a fraction of extraction power of MeOH – 27% and 33%, respectively. Acetone demonstrated relatively acceptable efficiency (55% of pure MeOH). The extraction efficiency in other solvents was extremely low, ranging from 0% effectivity of hexane, toluene and EtOAc (ethylacetate) to 35% of MeCN (acetonitrile).

The first extraction step accounted for a 60% – 75% of total effectivity for most of the tested solvents and second step delivered quite stable 20% - 25% in majority of cases. Only in solvents which exhibited lower extraction power in first step (MeCN, acetone, THF), higher amount of muscotoxin (30% - 35%) was extracted in the second one. Generally, third extraction step delivered not more than 5%, but in cases of solvents containing prevalence of water (30% MeOH, 5% acetic acid, H_2O) or some others like DCM (dichloromethane), THF (tetrahydrofuran) or acetone it can reached up to 15%.

Regarding extraction time (data not shown), no prominent differences were revealed. Whether extraction proceeded for 60 or 120 minutes, it had only a slightest influence concerning final efficiency.

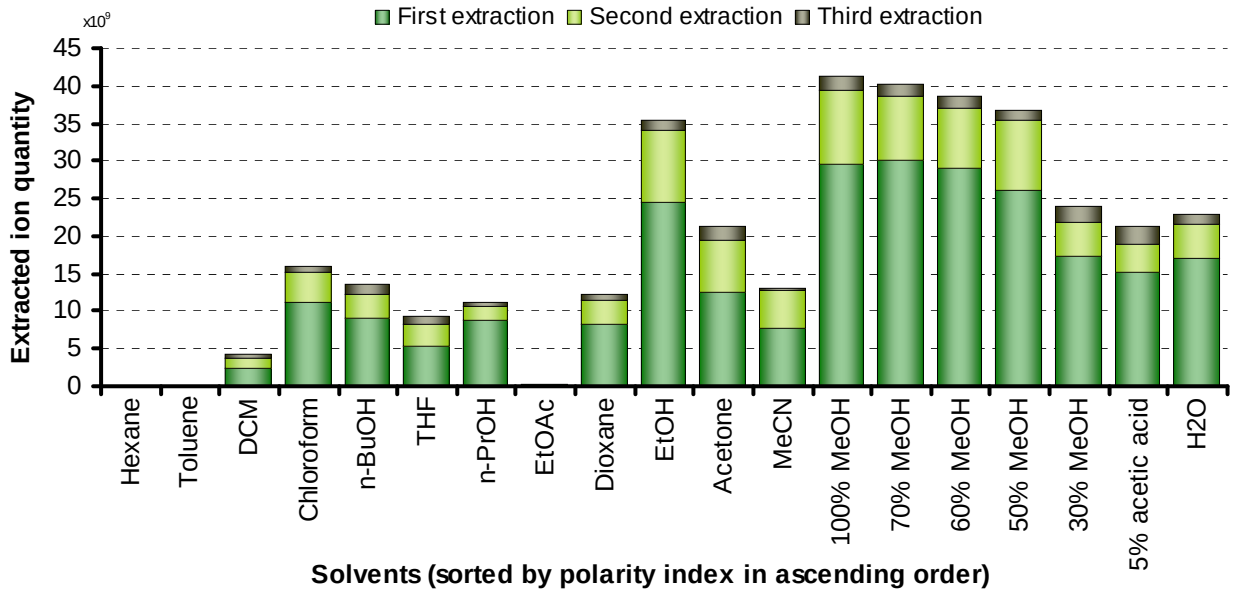


Figure 3.2. Effectivity of 19 organic solvent in extraction of muscotoxin in three consequent extraction steps. Abbreviations stand for following: DCM = dichloromethane, *n*-BuOH = *n*-butanol, THF = tetrahydrofuran. *n*-PrOH = *n*-propanol, EtOAc = ethylacetate, EtOH = ethanol, MeCN = acetonitrile, MeOH = methanol. Ion quantities on Y-axis are expressed in milliards of ions.

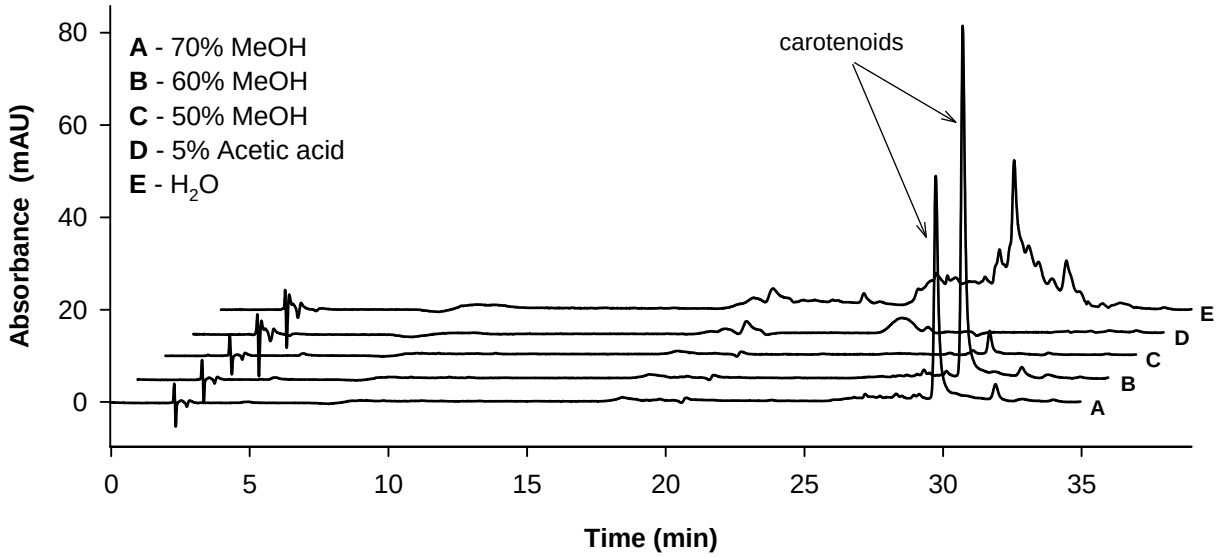


Figure 3.3. HPLC-UV chromatogram of five extracts (A-E) recorded at 440 nm wavelength. Marked peaks at Rt 29 min correspond to presence of carotenoids.

As the extraction efficiency is not the only discriminative factor favouring the potential solvent of choice among others, content of impurities (prevalently carotenoids and chlorophylles) plays also an important role. Thus, presence of these compounds which could hamper further purification steps has been examined.

The ratio of pigment versus compound content was enormously high in all of the nonpolar extracts (hexane, toluene, DCM, chloroform, *n*-BuOH, THF, *n*-PrOH, EtOAc, dioxane, EtOH, acetone, MeCN, 100% MeOH) (Fig. 3.2). Significantly lower ratio was found at potentially more suitable polar solvents present at the right portion of Fig. 3.2. (70%-30% MeOH, 5% acetic acid and H_2O). The pigment presence among these extracts was highest in 60% MeOH, followed by 70% MeOH and water. Only trace amounts were observed in 50% MeOH extract and 5% acetic acid (Fig. 3.3). These results correlates well with visual evaluation of extracts´ colour intensity.

Eventually, no ideal solvent with high extraction power and absence of carotenoids and chlorophylles was found because at least trace amounts of pigment contamination were found in every extract. Thus, my next objective was to develop a prepurification method to selectively remove pigments from the extract by partitioning.

The most effective partitioning solvent was EtOAc, retaining 80% - 85% of muscotoxin at the ratios where the EtOAc volume is equal to the proportion of MeOH in the original extract and with addition of excess of water (Fig. 3.4). At elevated levels of EtOAc at ratio 1:1:2 (extract:EtOAc:water) the partitiong was severely hampered in all cases and retained only 48%, 56% and 62% of the compound from the 50%, 60% and 70% MeOH extracts, respectively. If the proportion of water was increased to 1:1:4 (extract:EtOAc:water) ratio the retention rises by 10% in the case of 70% MeOH extract. Increasing EtOAc proportion to 1:3:2 (extract:EtOAc:water) ratio resulted in lowering the compound retention to minimal 21% and incrementing water share

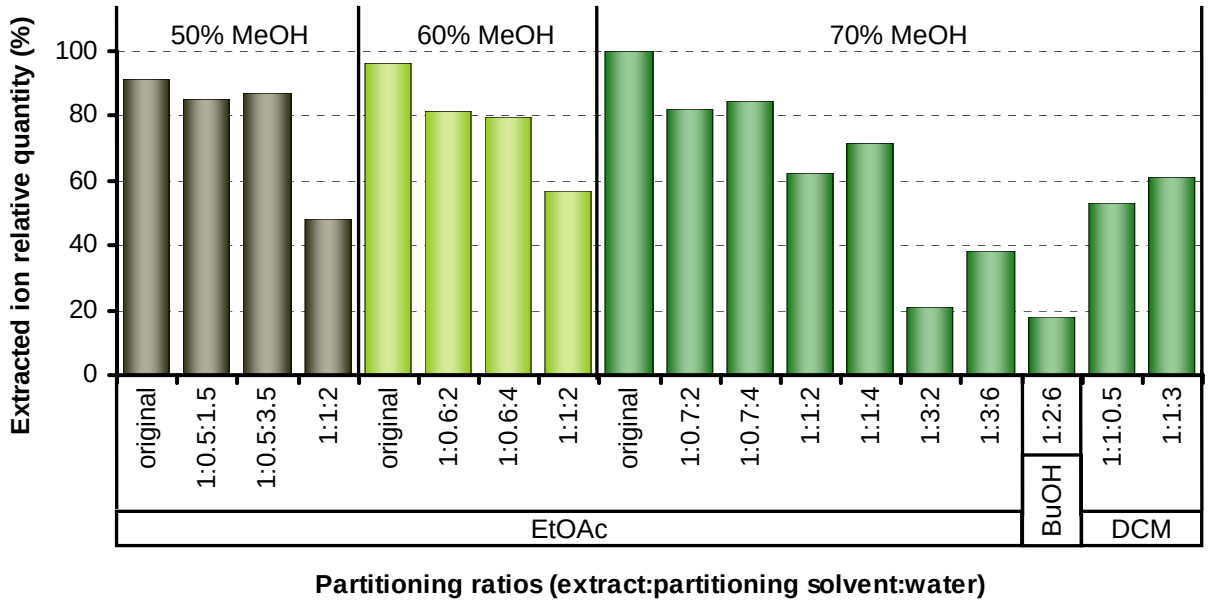


Figure 3.4. Partitioning efficiency of three solvents (EtOAc, BuOH, DCM) in retention of muscotoxin. Original extracts (50%-70% MeOH) for partitioning are denoted at the top part of Figure. Organic solvents used for partitioning are described under the Figure. Ratios under the columns stand for the final mixtures of solvents in following order (original extract:partitioning solvent:water - from bottom to top). Percentage values of every treatment represent total content of muscotoxin in extract before/after treatment normalized to the 70% MeOH original extract value (100%). original = original extract before partitioning.

Figure 3.5. Completed partitioning of 50% MeOH extract in ratio 2:1:3 (extract: EtOAc:water) right before collecting the lower (pink) phase.

to 1:3:6 (extract:EtOAc:water) ratio again rose the effectivity by 18%. The final of phases after partitioning was equal to ((*Total volume – 0.5* V_{EtOAc}) / *0.5* V_{EtOAc}) in all described treatments.

Other partitioning solvents were unable to reach the efficacies of EtOAc. BuOH displayed only 19% retention at ratio 1:2:6 (extract:BuOH:water) and DCM slightly higher value of 51% and 62% at 1:1:0.5 and 1:1:3 (extract:DCM:water) ratios, respectively.

Toluene partitioning couldn´t be performed at any ratio due to formation of dense milky emulsion. Emulsion was problem during all partitionings, not only toluene but diluting the original extract to 10 mg/ml (biomass/solvent) and keeping it at 4°C prior to partitioning process for 1 day proved to be a good way to notably reduce the effect. Emulsification was more pronounced when partitioning 100% MeOH extract compared to others. The lowest level of emulsification was found when processing 50% MeOH extract. Altough carotenoids and chlorophylles were removed completely (Fig. 3.6), extract wasn´t colorless and displayed light pink tinge (Fig 3.5). Furthermore, as the loss of the compound was still significant (10 %) I decided to refine the

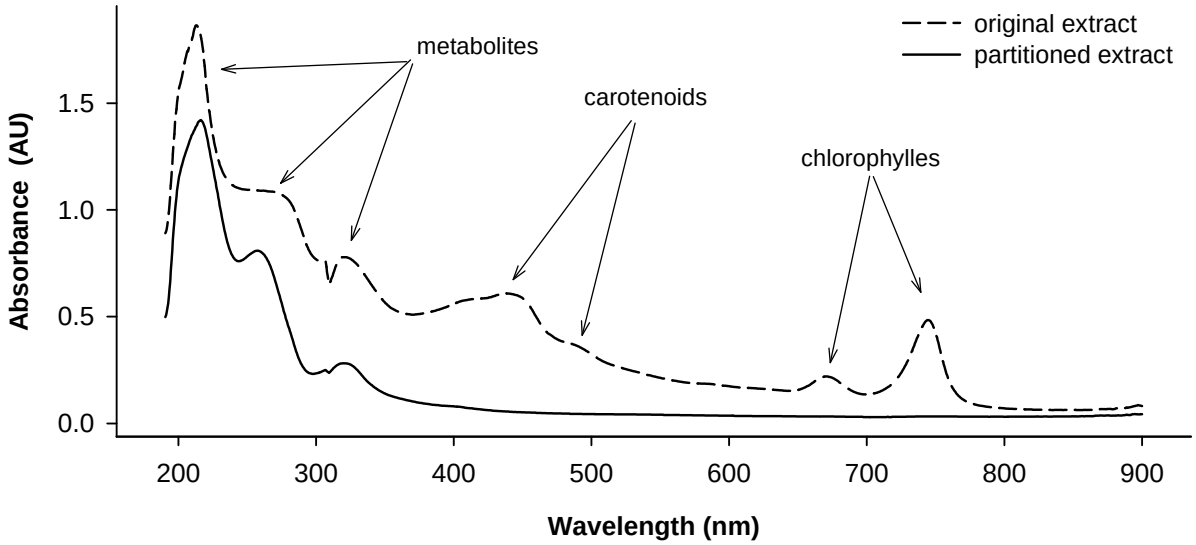


Figure 3.6. Absorption spectra of 50% MeOH extract before and after partitioning in ratio 2:1:3 (extract:EtOAc:water). Peaks are denoted by its anticipated constituents.

process further by performing second partitioning. Eventually, 99% of original compound content were recovered.

Such prepurified extract had to be concentrated on SPE (solid-phase extraction) columns prior to chromatographic purification. But when it was eluted through the columns, and adsorbed compounds washed out with 100% MeOH, HPLC-MS analysis revealed that eluate didn´t contain the muscotoxin. Paradoxically, it wasn´t detected even in eluted original extract (data not shown). Apparently, the problem hampering the routine SPE concentration step was residual EtOAc or excessive amount of MeOH. Thus, I have examined the effect of EtOAc and MeOH on HLB cartridge retention.

Firstly, there was a need to develop method to examine at least approximate quantitative presence of both components in prepurified extract. I have started by preparing a dilution series of MeOH and EtOAc and comparing their absorbance spectra to the partitioned extract. According to the expectation that no compound in the extract would absorb in the wavelengths longer than 230 nm, the shoulder at 245 nm was assigned to the presence of EtOAc. Comparing the absorption spectra of prepurified extract and blank dilution series of EtOAc it was easy to calculate approximate concentration of EtOAc in prepurified extract to be ~13.6 % (Fig. 3.7). Absorption spectra of MeOH dilution series and extract was overlapping, thus it was impossible to determine content of MeOH directly. But assuming no water is present in EtOAc phase (top phase after partitioning) (Fig 3.5) and final phase ratio of partitioning process is 11:1, it was assumed that MeOH content should be equal in proportion to EtOAc making final ratio of constituents in prepurified extract 1:1:5.3 (MeOH:EtOAc:H_2O).

According to these presumptions, blank mixture of solvents was prepared and its absorption spectra recorded before and after evaporation. Indeed, results proved that calculated ratio is probably real value and more importantly, that 60 minutes of evaporation at 37°C is adequate to eliminate EtOAc and lower the MeOH content to appx. 2.5% (Fig. 3.7).

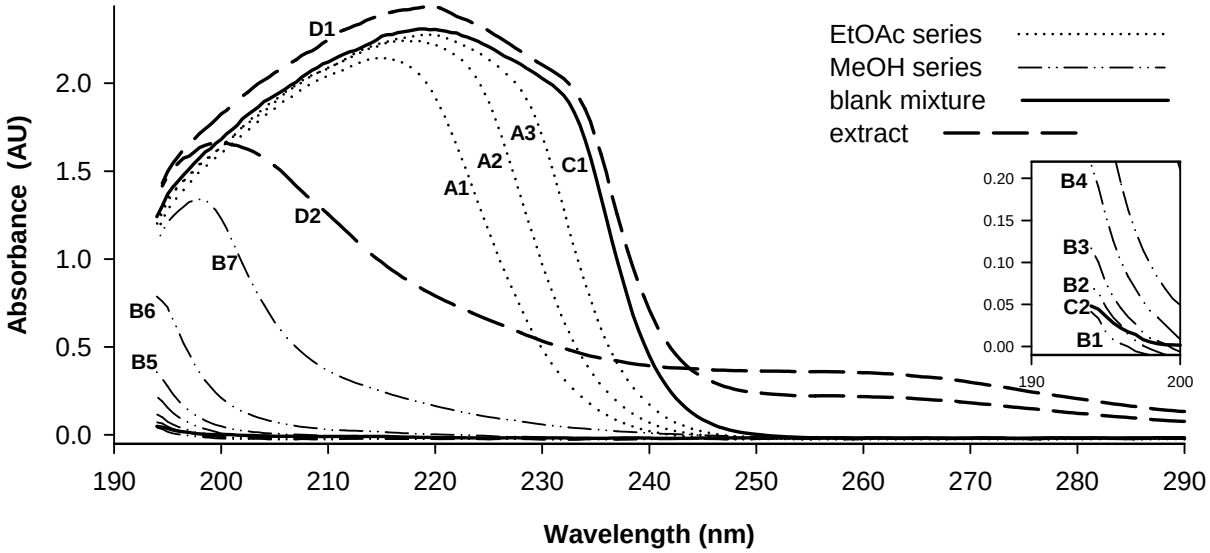


Figure 3.7. Absorption spectra of MeOH and EtOAc dilution series, 50% MeOH extract and blank mixture of solvents before and after evaporation. Square box in the right portion of the figure is magnified left-end portion of the plot. To identify the spectra, legend serves as discriminator for type of experiment and alphanumerical values further specify it as followed: A1 = 1.25%, A2 = 2.5%, A3 = 5%; B1 = 1.25%, B2 = 2.5%, B3 = 5%, B4 = 10%, B5 = 20%, B6 = 50%, B7 = 100%; C1 = blank mixture before evaporation (1:1:5.3) (MeOH:EtOAc:H_2O), C2 = blank mixture after 30 min evaporation; D1 = 50% MeOH partitioned extract before evaporation, D2 = 50% MeOH partitioned extract after 30min evaporation.

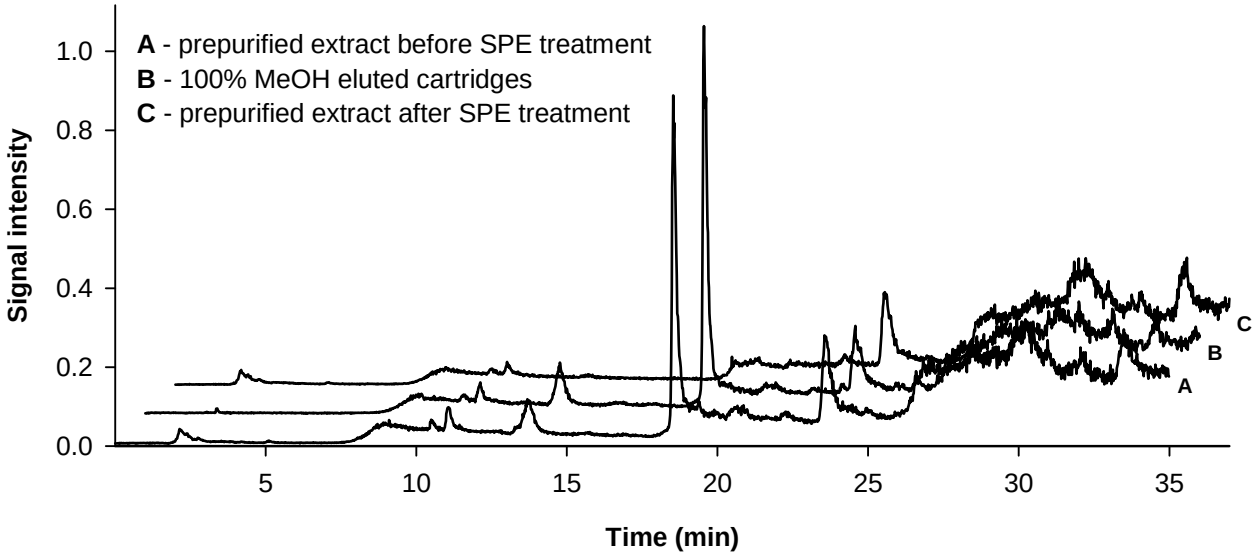


Figure 3.8. HPLC-MS chromatograms (TICs) of 50% MeOH partitioned extract during the SPE concentration process. Prepurified extract **(C)** can be interpreted also as nonadsorbed eluate. 100% MeOH eluate **(B)** was diluted to the level of prepurified extract before SPE treatment **(A)** prior to HPLC-MS analysis.

Such protocol was applied to real extract and subsequent SPE was carried out with no problems. During the process, only minor fraction of active compound was lost (1.2%) (Fig. 3.8) and additionally, pink contaminant remained in the non-adsorbed waste eluate (visual observation). After this treatment, it was possible to concentrate extract up to 1.2 g of cyanobacterial biomass/ml of solvent with no colloidal or precipitated particles.

Muscotoxin present in highly concentrated prepurified extract was subjected to fractionation by preparative HPLC in order to separate it from other metabolites in the extact. Thus, I have been developing optimal separation parameters including gradient, solvents and suitable column. The most optimal gradient was shown and described in methodics part (Fig. 2.1. P_1). Using this gradient the muscotoxin was relatively well-resolved and separated from other compounds visible in UV detection. muscotoxin was eluted at Rt range 36.5 – 38.5 min exhibiting inconvenient tailing. Thus, fraction was collected at the plateau of the peak only (Fig. 3.9. Top). Substances *m/z* 886, 900 and 1098 were eluted in Rt 10 – 20 min and *m/z* 1112 was present in three Rt – 23, 28 and 32 min. Previously undetected compounds *m/z* 1128 and *m/z* 1226 were eluting right at the base of muscotoxin´s peak at Rt 35.5 min and Rt 40 - 41.5 min, respectively. Unfortunately, the purity was not optimal after first separation and prevalently compounds with *m/z* 1112, 1128 and 1226 as well as unidentified yellow substance(s) spoiled the resulting product (data not shown).

Subsequent purification step was needed and as C18-RP sorbent proved to be effective in separation, the development of next step was focused on optimizing gradient and/or mobile phase composition. MeCN:MeOH (9:1) and H_2O was selected as the most effective mobile phase composition for the next separation step. Gradient and elution parameters had to be completely redesigned (Fig. 2.1. P_2), but column sorbent remained the same, only diameter was increased to 25 mm. This separation step provided sufficient resolving efficiency and all peaks displayed ideal chromatographic shapes (Fig. 3.9. Bottom). Muscotoxin eluting in Rt 51.5 min was

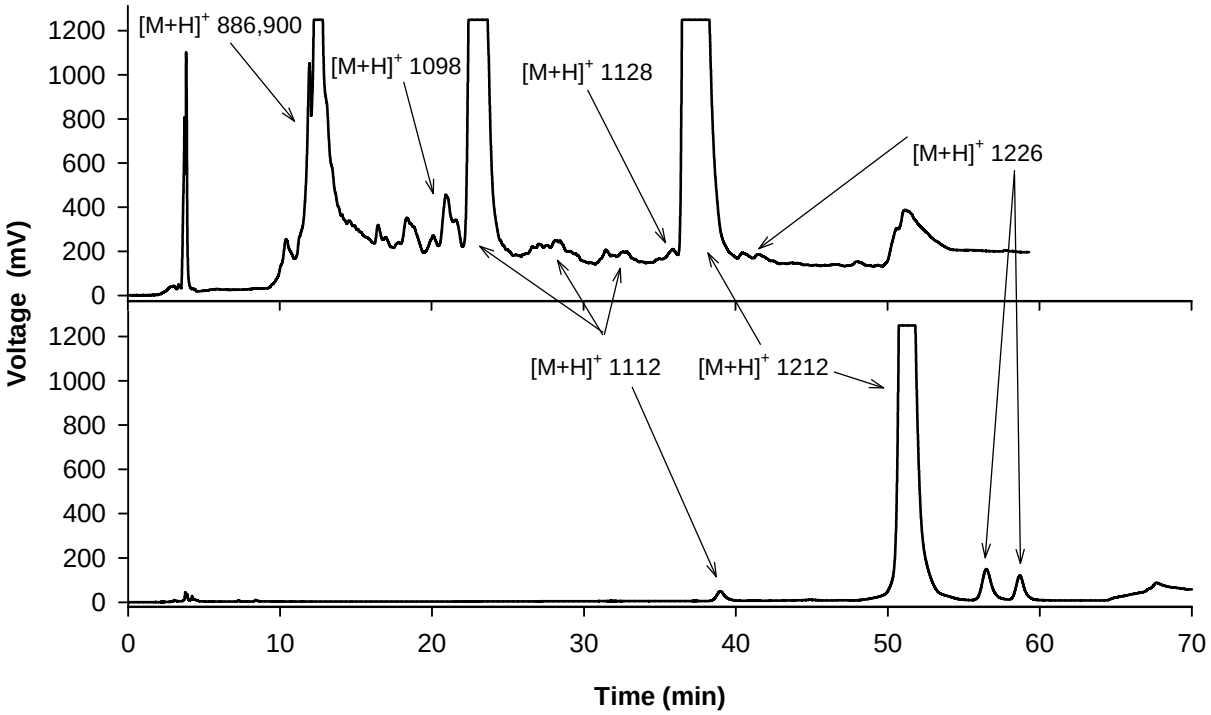


Figure 3.9. **Top:** Preparative HPLC chromatogram of prepurified 50% MeOH extract acquired using gradient P_1 (refer to methodics section 2.1.3 for details)
Bottom: Preparative HPLC chromatogram of fraction $[M+H]^+$ 1212 from previous separation acquired using gradient P_2 (refer to methodics section 2.1.3 for details)
Depicted *m/z* values stands for ions with maximum observed intensity after analysis on HPLC-MS.

completely resolved from accompanying compounds (*m/z* 1226 - Rt 56 and 58.5 min and *m/z* 1111 - Rt 39 nd 44 min). At Rt 67 min there were still traces of some unidentified contaminants confirming the necessity of second step. The accompanying compounds were also collected in the process.

Both of them displayed similar elution pattern as the active compound. Main peak of these two compounds with highest intensity was always followed by well-resolved minor peak(s) containing compound of same molecular mass as can be found in parent peak (Fig. 3.10). Additionally, HPLC-UV/DAD-MS analysis revealed that individual structures displayed overlapping retention at same chromatographic conditions. Muscotoxin fraction (*m/z* 1212) contained at least two recognizable congeners of same mass eluted at Rt 18.7 (main peak) and 21 min. Fraction with compound of *m/z* 1112 contained three congeners eluted at Rt 15.5 min (main peak), and 18.7 and 21 min being congruent with muscotoxin analysis and the last fraction (*m/z* 1226) very precisely mirrored the analysis of muscotoxin and its congeners eluted also at identical Rt (Fig. 3.10).

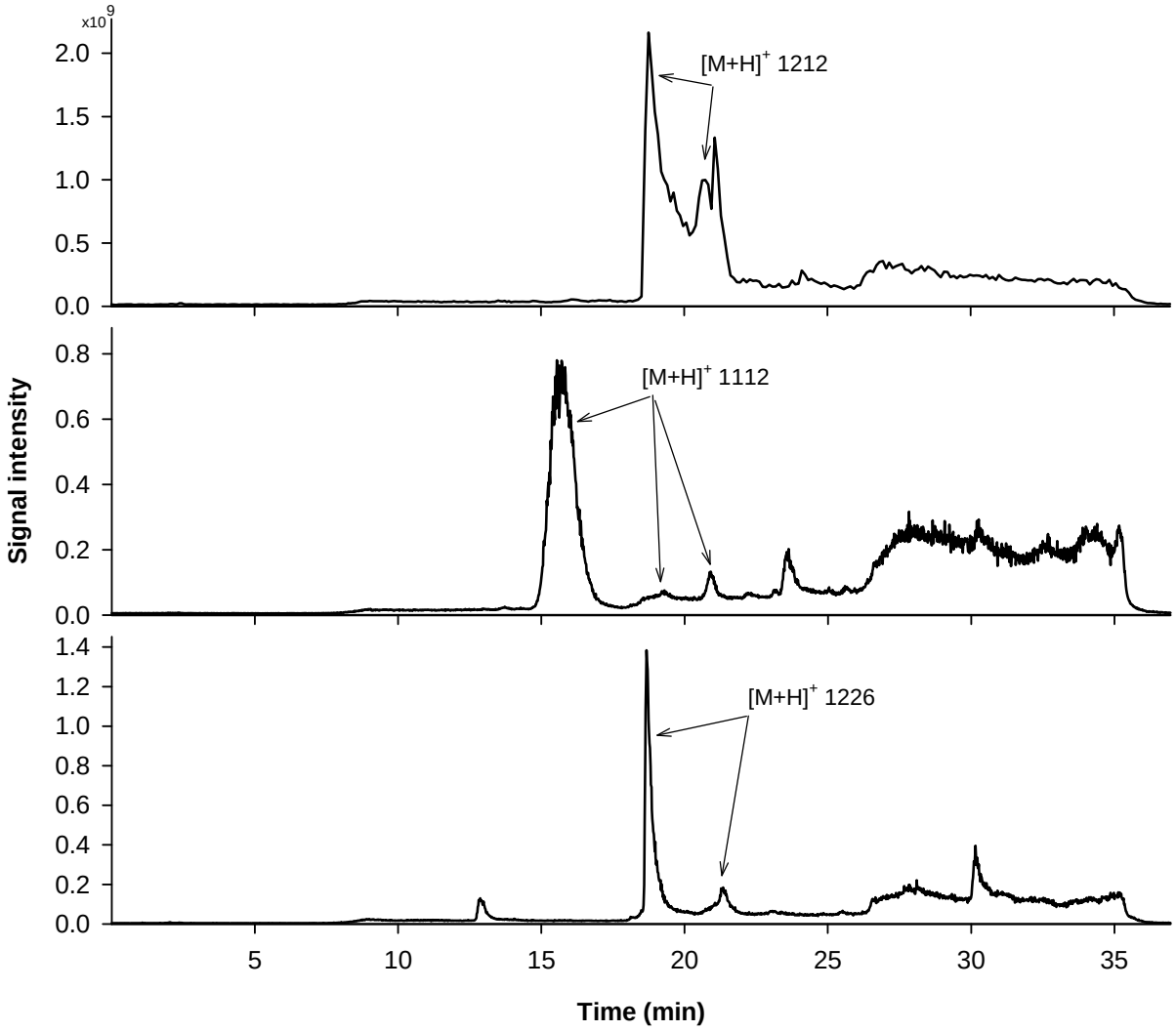


Figure 3.10. HPLC-MS chromatograms (TICs) of fractions acquired during preparative HPLC separation on Fig. 3.9. at **Top:** Rt 37 -38 min. **Middle:** Rt 22 -23 min. **Bottom:** Rt 41 -43 min. Denoted *m/z* values stands for the most intensive mass at given Rt.

Both congeners with *m/z* 1212 and *m/z* 1226 eluting at Rt 18.7 and 21 min (Fig. 3.10. Top and Bottom, respectively) were collected during analyses in order to check the efficiency of their separation. After evaporation and resuspending of fractions in original injection volume, the analyses were repeated at same chromatographic and MS conditions. Analyses of both congeners present in analyses of *m/z* 1212 and *m/z* 1226 (collected at Rt 18.7 and 21 min) resulted surprisingly in chromatograms identical to the one they were collected from (data not shown).

Observing the mass spectra of all three structures, the similar fragmentation patterns were observed (Fig. 3.11.). $+MS^2$ fragmentation of quasimolecular ion *m/z* 1211.7 displayed abundant neutral losses of water forming ions *m/z* 1157.6, 1175.6 and 1193.7. Fragment ions *m/z* 928.5, 910.6 and 433.2 were another very stable products in the spectrum. After thorough observation of $+MS^2$ spectrum of quasimolecular ion *m/z* 1225.7, we can find that it is very similar with the spectrum of *m/z* 1211.7 with exception that fragment ions are heavier by the difference in the parent ions (*m/z* 14). On the other hand, fragmentation spectrum of ion *m/z* 1111.6 didn´t show

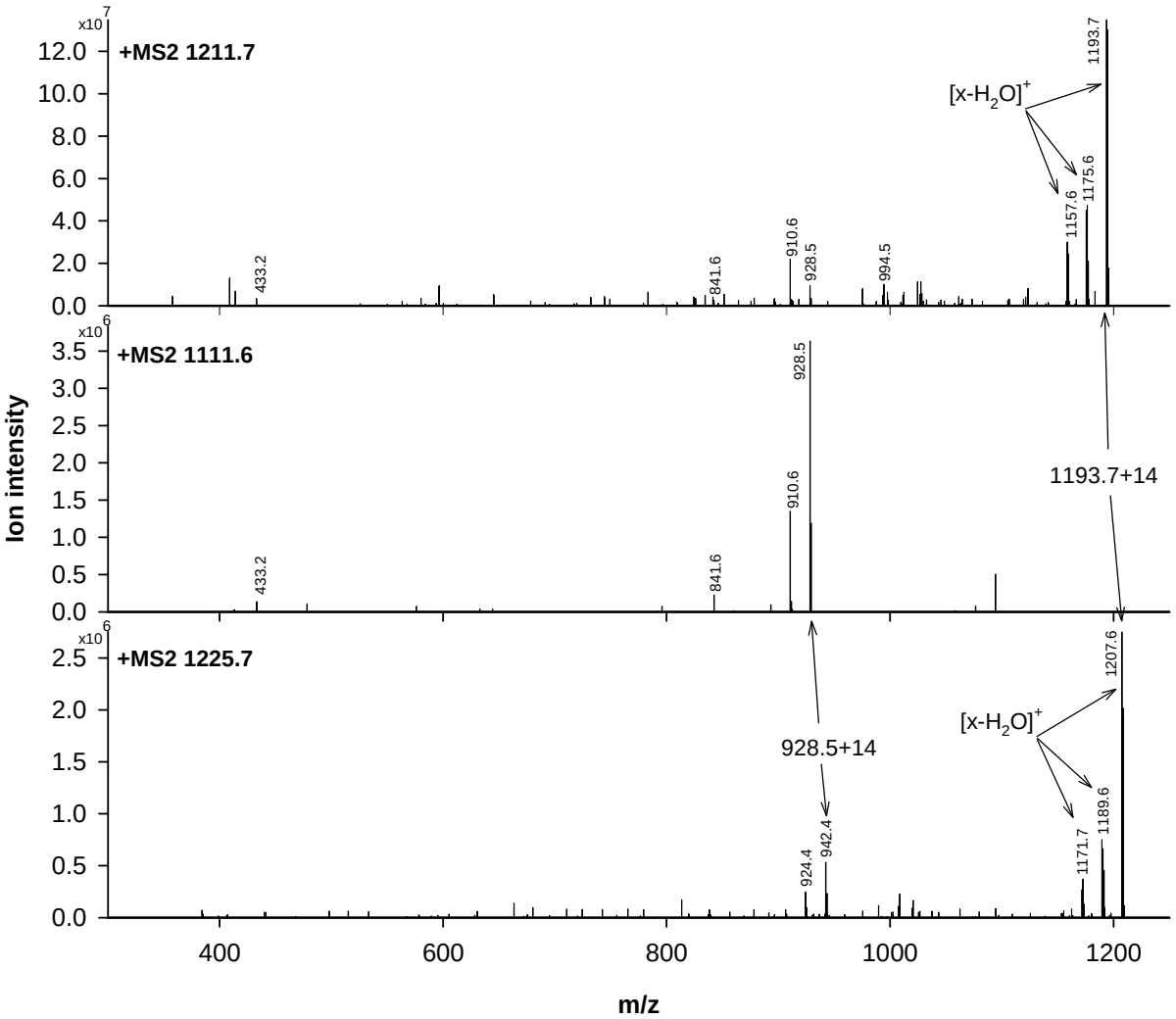


Figure 3.11. $+MS^2$ fragmentation spectra of quasimolecular ions present in mass spectra of analyses displayed on Fig. 3.10. **Top:** *m/z* 1211.7 from mass spectrum of analysis Fig 3.10. Top (Rt 19 min). **Middle:** *m/z* 1111.6 from mass spectrum of analysis Fig 3.10. Middle (Rt 16 min). **Bottom:** *m/z* 1211.7 from mass spectrum of analysis Fig 3.10. Bottom (Rt 19 min). Captions denote the residues between individual fragments.

neutral losses of water but identical fragment ions *m/z* 433.2, 841.6, 910.6 and 928.5 can be found.

Compound was eventually obtained as an amorphous white powder at HPLC purity level (~99%) with approximate yield of 2 mg/1 g (muscotoxin/biomass).

According to FT-MS measurement ($[M+H]^+$, m/z 1211.6666, appx. 1 ppm error), the molecular formula was determined to be $C_{58}H_{90}N_{12}O_{16}$. Absorbance maximum was detected at 227 nm. Compound was soluble in MeOH only to some extent but perfectly dissolved in DMSO. Thus, the substance was stored either dissolved in DMSO or in a dry form at -20°C for further experiments.

Muscotoxins molecular structure were determined by both NMR experiments and MS fragmentation analyses. Individual structural subunits of molecule were determined by NMR spectroscopy. Amino acid residues spin systems were determined by extensive application and combination of 2D spectral method (COSY, TOCSY and 1D-TOCSY). Individual carbon-hydrogen signal correlation and their multiplicity were assigned using proton-edited HSQC. Quarternary signals were detected by HMBC experiments.

It was found out that structure of muscotoxin is comprised of 11 subunits in a cycle (Fig. 3.12). The subunits were determined to be standard amino acids glycine, serine, isoleucine (2x),

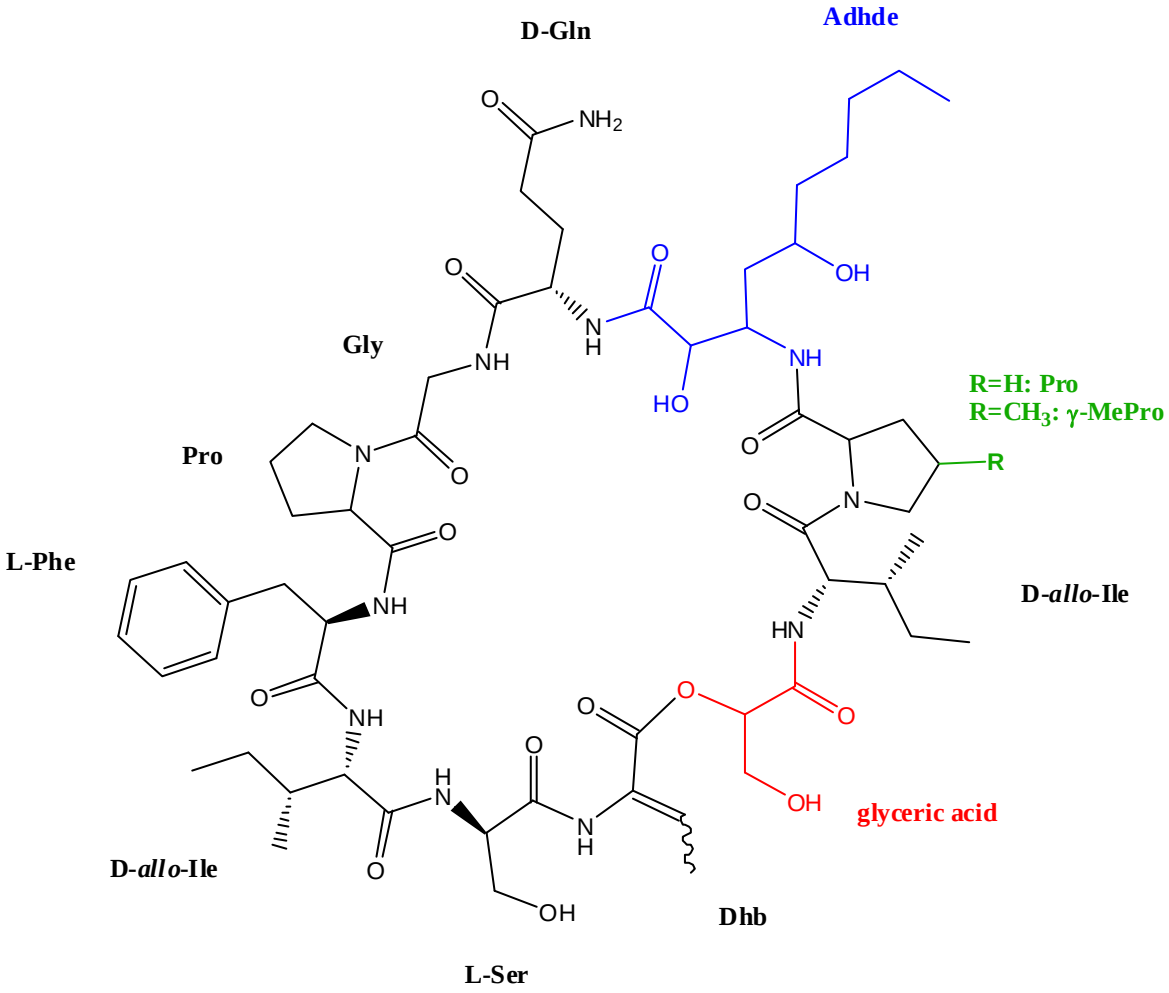


Figure 3.12. Structure of muscotoxin (R = H) and its congener *m/z* 1225 (R = CH_3)

glutamine, phenylalanine and proline (2x). Additionally, structure was found to contain one nonstandard amino acid Dhb (2,3-dehydro-2-aminobutyric acid) and one unique β-amino acid Adhde (3-amino-2,5-dihydroxydecanoic acid). Furthermore, structure implements one glyceric acid connected via depsipeptide bond to Dhb aminoacyl. One Pro subunit was discovered to be substituted by γ-MePro in its congener *m/z* 1225. Exact location of substituted γ-MePro has not been determined yet.

Subunits sequence in molecule was determined using high resolution MS fragmentation techniques. Sequence was partially determined by NMR and based on these results, several probable theoretical masses of a- and b- fragment ions (including losses of water and ammonia) were generated. These theoretical ions were then compared with deconvolated masses acquired from high ress MS spectra. Position of Pro in the cycle and complete sequence were determined according to the highest congruence of fragments. Higher probability of ring opening at N-terminus allowed to determine the orientation of the fragments. It was confirmed by NMR spectroscopy in HMBC spectrum. Amide linkage of Adhde β-amino acid was assigned likewise by HMBC correlations.

Derivatization of acid hydrolysate and comparing the GC-MS chromatograms with appropriate amino acid standards revealed following configuration of amino acids: D-*allo*-Ile, Gly, L-Ser, (?)Pro, D-Gln, L-Phe. Three peaks in the TIC chromatogram remained unidentified which apparently correlated to the rest of the three nonstandard subunits identified by NMR.

3.2. CELL TOXICOLOGY AND MECHANISM OF ACTION

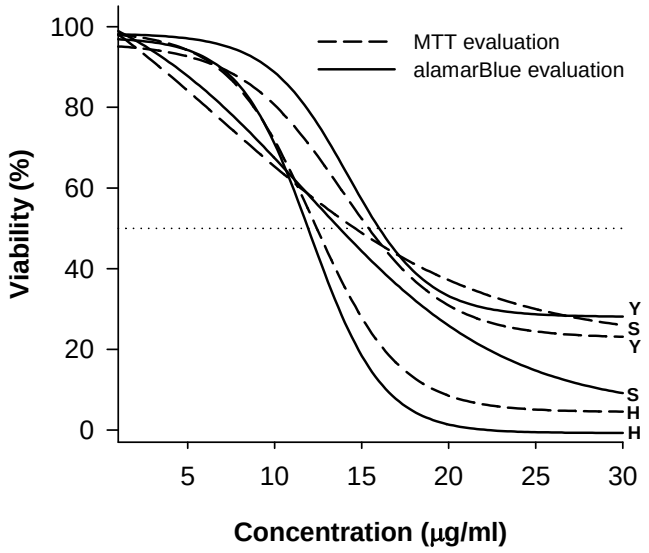


Figure 3.13. Dose-response curves of three cell lines obtained using two evaluation assays. Cell line abbreviations stands for following: Y = YAC-1, S = Sp2, H = HeLa. Dotted line intersects 50% viability value (IC_{50}).

The first step in characterization of toxic effect of muscotoxin was to evaluate its potency. Two similarly based assays (alamarBlue and MTT) served for parallel evaluation and confirmation of the results. After determining the viability of each cell line treated with dilution series of muscotoxin and fitting the appropriate dose-response curve, following IC_{50} values were calculated by regression analysis with 95% credibility values. The highest concentration of muscotoxin required to inhibit cell growth to 50% was observed in the case of YAC-1 cells, which IC_{50} was found to be 16 and 15.4 μg/ml evaluted by alamarBlue and MTT assay, respectively

(Fig. 3.13). Sp/2 cells required lower concetration on average to achieve the same effect, IC_{50} = 13.7 and 16.4 μg/ml in same order of assays. Finally, HeLa cells were found to be most susceptible with IC_{50} = 11.9 and 12 μg/ml determined by alamarBlue and MTT, respectively.

Both sets of dose-response curves obtained by using different evaluation assays fairly correlated with exception of Sp/2 cells plots which differs in highest concentrations (Fig. 3.13). Toxin was unable to reach IC_{100} in the case of non-adherent cell lines even at 30 μg/ml concentration which completely inhibited growth of HeLa cells (IC_{100} > 23 μg/ml). HeLa dose-response curve evaluated by MTT was unable to reach IC_{100} at all. On the other hand, at lower concentrations Sp/2 cell line seems to be most susceptible and 5 μg/ml of muscotoxin induced measurable changes in viability, as opposed to other two cell lines.

As the range of active concentrations was determined, muscotoxin was subjected to experiment monitoring toxic effect dynamics based on modified alamarBlue assay. Due to the fact that phenotype of HeLa cells treated by muscotoxin was reminiscent of detergent effect, Triton X-100 was evaluated together with it throughout following experiments.

Evaluation of results revealed that toxic effect of muscotoxin at highest concentration (30 μg/ml) was indeed rapid and fatal, and from the initial insult throughout the end of incubation period there was practically no change in absorbance, which suggested death of all cells exactly after muscotoxin addition (Fig. 3.14. Top left). At 20 μg/ml the onset of toxic effect was also in the beginning but lowered dose prolonged reaching the zero metabolic activity to appx. 15h of incubation. The toxic effect at further dilutions was manifested as undirectly proportional function of toxin concentration and time but absorbance rose in same manner up to the point (5 μg/ml) where only minor effect could be recognized.

The kinetics was much better visualized at bottom plot where Y-axis stood for "reduction rate" (Δ_A) (Fig. 3.14. Bottom). It was observed that at highest dose, except the small rise in the beginning, the reduction rate was clearly zero. At concentration 20 μg/ml the onset of toxic effect was at 1 h after incubation, at 15 μg/ml its inception was delayed to 2 h, at 10 μg/ml first observable changes were at 6.5 h and in the case of 5 μg/ml at 12 h. Except from 5 μg/ml treatment all diluted series displayed similar shape of the plot. At appx. 6 h the reduction rate started to decrease linearly in dilution series 10-20 μg/ml and after next 10 h, 20 and 15 μg/ml treatments halted at 0.001 and 0.007 reduction rate while 10 μg/ml still continued its linear decreasing trend. Control cells kept steady reduction rate 0.02 from 5-20 h.

Kinetics of toxic effect of Triton X-100 largely resembled that of muscotoxin (Fig. 3.14. Top right). Altough toxic effect was characterized as rapid and similar to detergent effect of Triton X-100, there wasn´t enough data to tell something about its mechanism of action. Thus, the effect of muscotoxin was observed via immunofluorescence and differential interference contrast (DIC) microscopy. Firstly, the fixed samples of HeLa and mouse fibroblast cells treated with muscotoxin were prepared and observed.

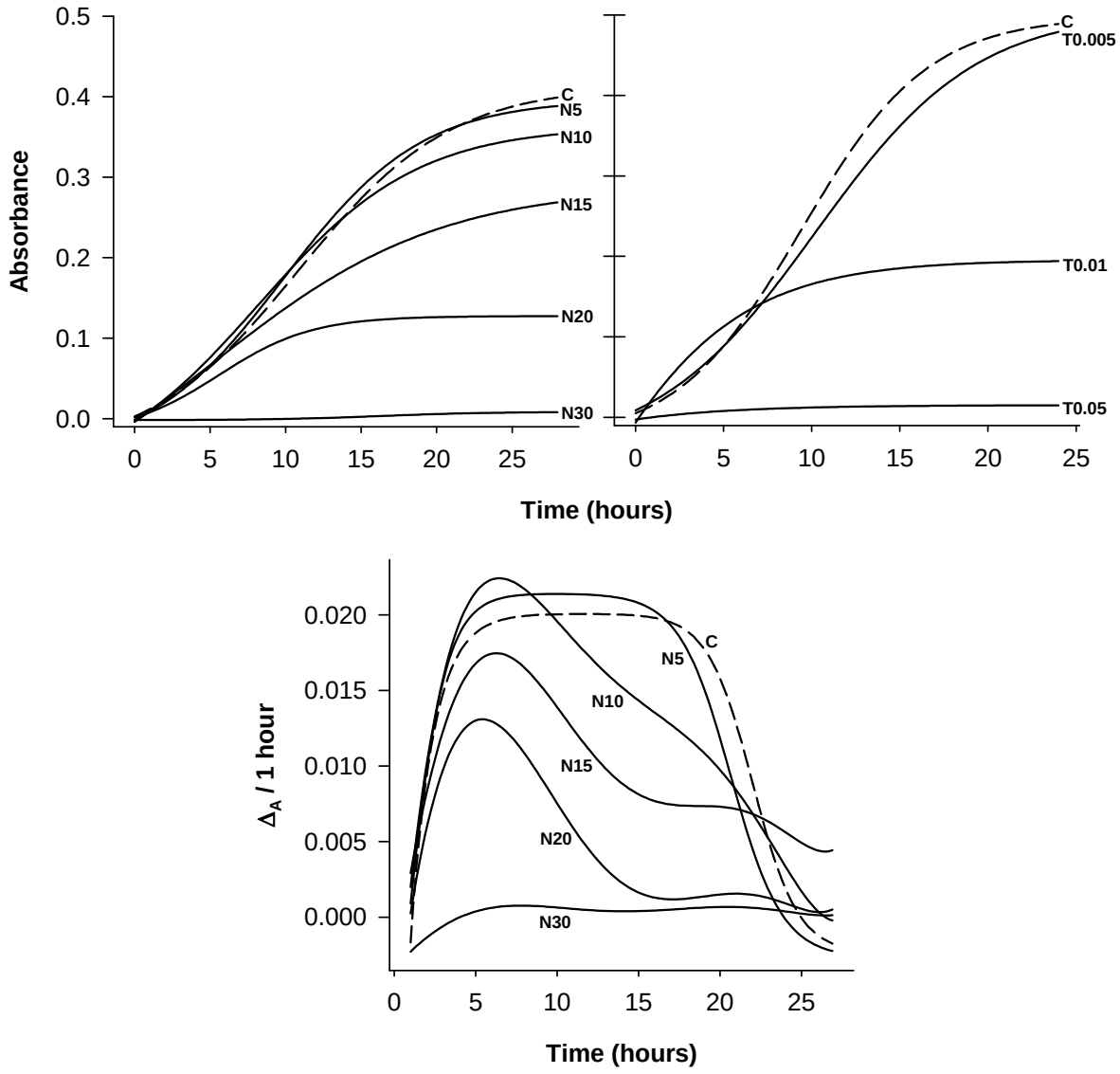


Figure 3.14. Absorbance development of the alamarBlue assay during treatment of HeLa cells with
Top left: muscotoxin
Top right: Triton X-100.
Plots were fit by dose-response curve.
Bottom: Absorbance rate development of the alamarBlue assay during treatment of HeLa cells with cytotoxin NM.
N5 and C plots were fit by bell-shaped curve, N10-N30 by 6th order polynomial.
Abbreviations stands for following: C = control nontreated cells, N = muscotoxin (numerical values in suffix reflect concentration in μg/ml), T = Triton X-100 (numerical values in suffix reflect concentration in %).

Both type of cells reacted to the treatment with muscotoxin by identical phenotype changes. After 1 h incubation with 25 μg/ml dose of cytotoxin, both cell lines exhibited drastic changes in morphology as observed in DIC (Fig. 3.15). Cell membrane was disrupted with clump-like aggregates, intense contraction resulted in change of flat-like morphology to protuberant one compressing the cell nucleus and cytoplasmic organelles. Cell shapes after insult was shrunk to a variable extent and were displaying random shapes. Focal adhesions were relatively intact and cells still maintained contact with coverslip. Effect was similar in

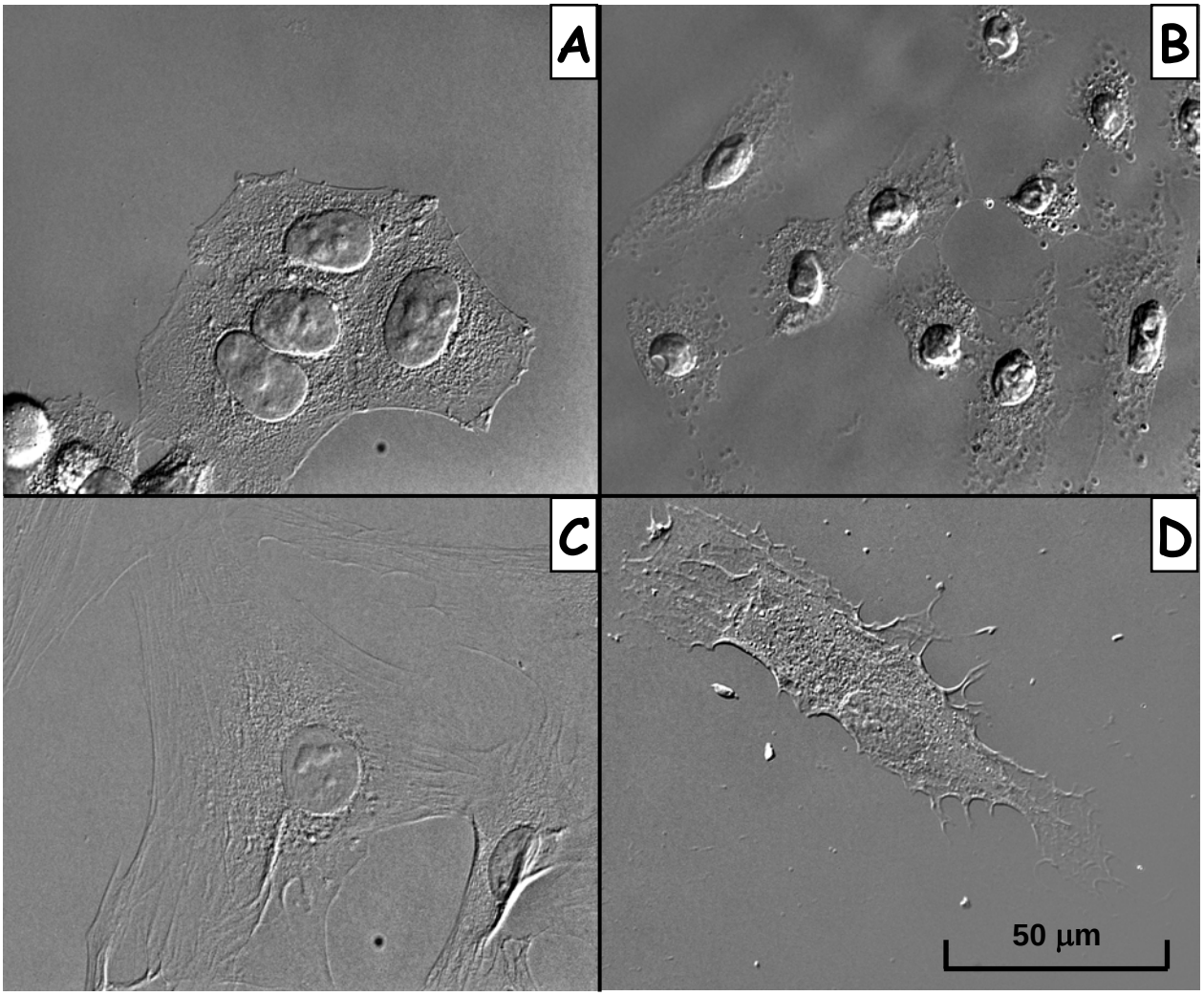


Figure 3.15. DIC images of HeLa (A-B) and mouse fibroblast (C-D) cells, nontreated (A,C) and treated with 25 μg/ml dose of muscotoxin for 1 h (B,D).

concentration range 15 – 30 μg/ml and only intensity of the effect and time of onset differed. The cells exposed to 5 and 12.5 μg/ml doses showed mere faint morphological disturbances even at prolonged time of the incubation (data not shown).

Observing fluorescently tagged fibroblast cells uncovered changes in the cell organelles giving closer insight into specificity of mechanism of action (Fig. 3.16). After 1 h treatment with muscotoxin at concentration 25 μg/ml the cells displayed consistent but irregular condensation of chromatin in the form of bright patches. Actin cytoskeleton showed no marks of pathological changes except from expected aggregation of cortical filaments at cell borders. Mitochondria, on the other hand, accomplished conspicuous morphological transformation. The native interconnected tubular organelles turned into a rounded and swollen fragments with occasional signs of uncoupling.

Triton X-100 treatment (0.01%) of the same duration demonstrated practically equivalent phenotype regarding nuclei. Actin cytoskeleton exhibited total loss of cortical filament in the center of the cells showing generally worse cytoskeletal structure. More pronounced change was observed in red channel (Fig. 3.16). Mitochondria was neither resembling the native shape in

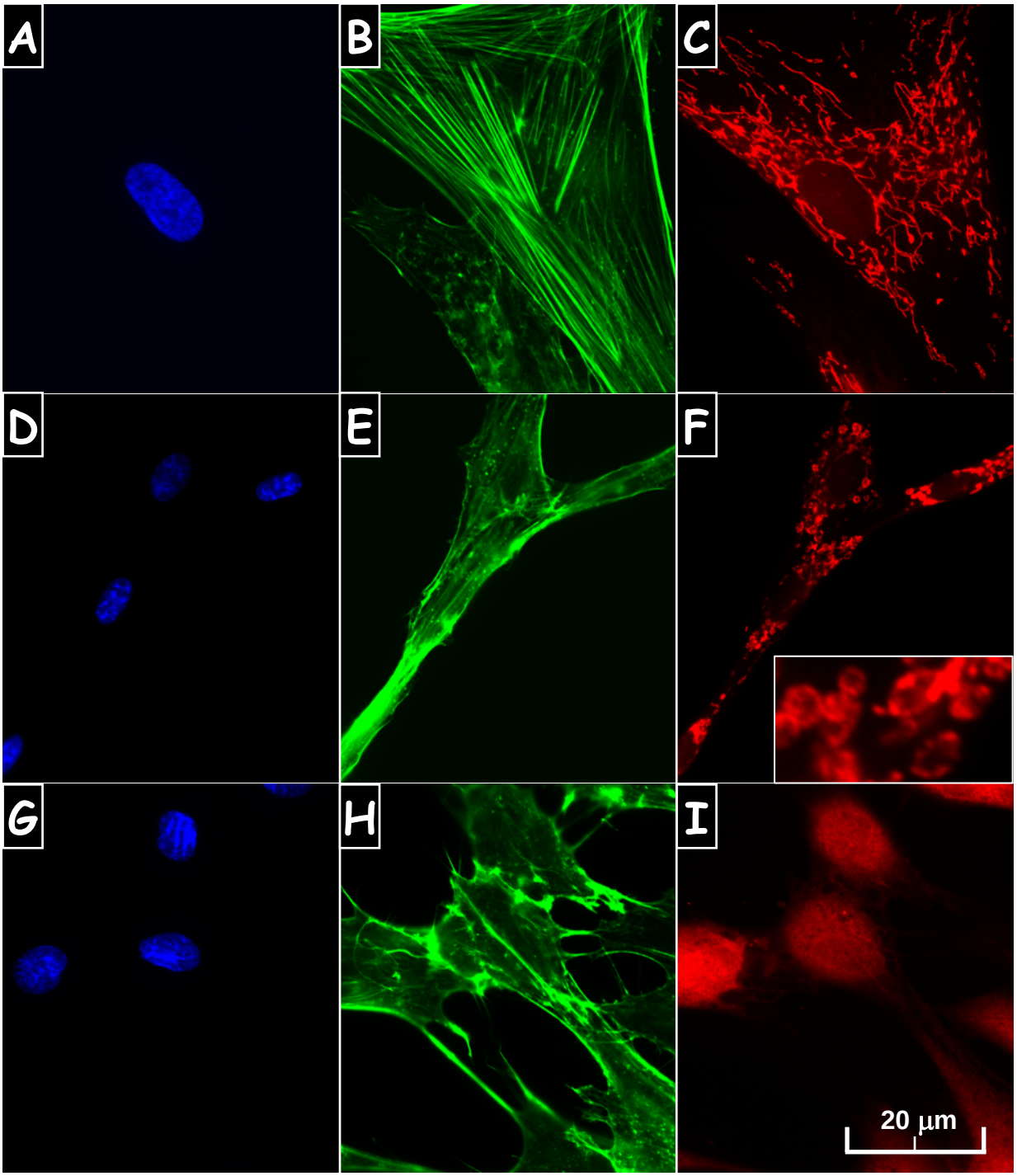


Figure 3.16. Fluorescent images of fibroblast cells, nontreated (A-C), treated with 25 μg/ml dose of muscotoxin for 1 h (D-F), and treated with 0.01% Triton X-100 for 1 h (G-I). Left column (A-G) = nuclei, middle column (B-H) = actin, right column (C-I) = mitochondria. Rectangle in image F contains enhanced section of central part showing circularised morphology of mitochondria.

healthy cell nor the perturbed morphology induced by muscotoxin but complete breakdown of mitochondrial integrity has been recorded. Only diffusive MitoTracker signal has been visible in the contracted cytosol.

As the kinetics of effect has been found to be rapid at high concentrations and morphological and mitochondrial abberations seems to be the key points, thus to observe the

initial stages of effect, it was decided to proceed with timelapse *in vitro* cinematography experiment observing isolated fibroblast cells treated with both sublethal and lethal doses. Cells were exposed to effect of 7 and 25 μg/ml doses of muscotoxin and observed in DIC and red MitoTracker channel simultaneously.

The observation of 25 μg/ml dose effect demonstrated extremely swift onset of membrane injury. Cells displayed first marks of injury as early as 40 seconds after addition of cytotoxin. In next 100 seconds membrane was completely eroded and cell has been vigorously contracting. No dramatic changes has been recorded in ensuing minutes and the cells only slightly continued in contraction and membrane exhibited pronounced tears in its structure. During whole timelapse experiment the cell was able to maintain contact with growth surface and has not been washed away. Paradoxically, focal adhesions at marginal protrusions were still relatively intact and carrying out its function properly.

Kinetics of mitochondrial disruption proceeded on similar time scale. After first 40 seconds mitochondria completely lost integrity, uncoupled and the signal was lost.

On the other hand, observation of 7 μg/ml dose effect revealed no notable morphological alterations during entire exposition time. Cells kept its native flat-like form. But mitochondria showed its pronounced susceptibility even at such low dose. Altough it took appx. 15 min to develop first marks of effect, mitochondria finally slowly started to fragment and lose its integrity.

Although it was morphologically proved that membrane suffered pronounced damage, quantitative data about the permeability was missing. Furthermore, it wasn´t clear whether muscotoxin interacts only with phospholipids or requires other membrane constituents e.g. carbohydrates or proteins as well for successful manifestation of the effect.

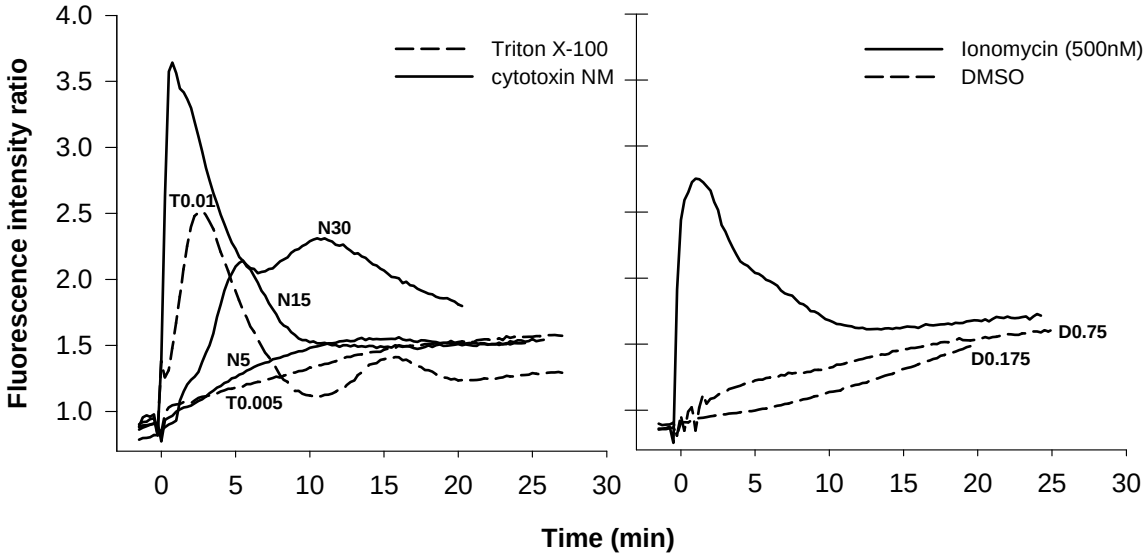


Figure 3.17. Fluorescence ratiometric measurement of cytosolic Ca^{2+} in HeLa cells treated with
Left: muscotoxin and Triton X-100.
Right: ionomycin and DMSO.
Abbreviations stands for following: N = muscotoxin (numerical values in suffix reflect concentrations in μg/ml), T = Triton X-100, D = DMSO (numerical values in suffix reflect concentrations in %).

Starting with measuring the kinetics of small-ion permeability it was found out that results were congruent with preceding experience (Fig. 3.17). At 30 μg/ml dose a rapid increase of intracellular Ca^{2+} ions $[Ca^{2+}]i$ in cytosol of HeLa cells in first minute after muscotoxin addition has been found. Subsequently, the decrease of $[Ca^{2+}]i$ to appx. one half of maximal intensity has been also observed. 15 μg/ml dose followed the same trend although the initial rise was somewhat delayed proceeding in appx. 5 mins. At sublethal 5 μg/ml dose there was only an inconspicuous rise in $[Ca^{2+}]i$ and comparing it to the effect of carrier solvent (0.175% DMSO) and the effect was thus negligible. Altough after subtraction of 0.175% DMSO plot, the minor ascent was recognizable at 5 mins followed by a decrease to inital 1.0 value which resembled the other two treatments.

Triton X-100 treatments resembled the muscotoxin and there were no noteworthy deviations. 500nM ionomycin has been used as the positive control in this experiment and it induced same rapid onset of $[Ca^{2+}]i$ leak as cytotoxin at highest dose and likewise followed decreasing course, although at much slower rate (Fig 3.17).

Membrane has been found to be permeable also for intracellular enzyme- lactate dehydrogenase (LDH) (Fig. 3.18. Left). At highest applied dose of cytotoxin (25 μg/ml) the effect manifested immediately after exposure resulting in 100% extracellular LDH. But then, the decrease of the value in 5 h to 70% and reaching ultimate 60% in 72h has been recorded. Such behaviour markedly reflected the $[Ca^{2+}]i$ measurement. At lower dose (10 μg/ml), LDH content increased at slower rate attaining 20% at 3 h exposition time and dropping back to initial 0% value in 5 h. Then, the extracellular concentration of LDH was only slowly rising up to the end of incubation period where it reached 10% (72h). 5 μg/ml dose exhibited only slight increase (2.8%) in extracellular LDH concentration at 48h.

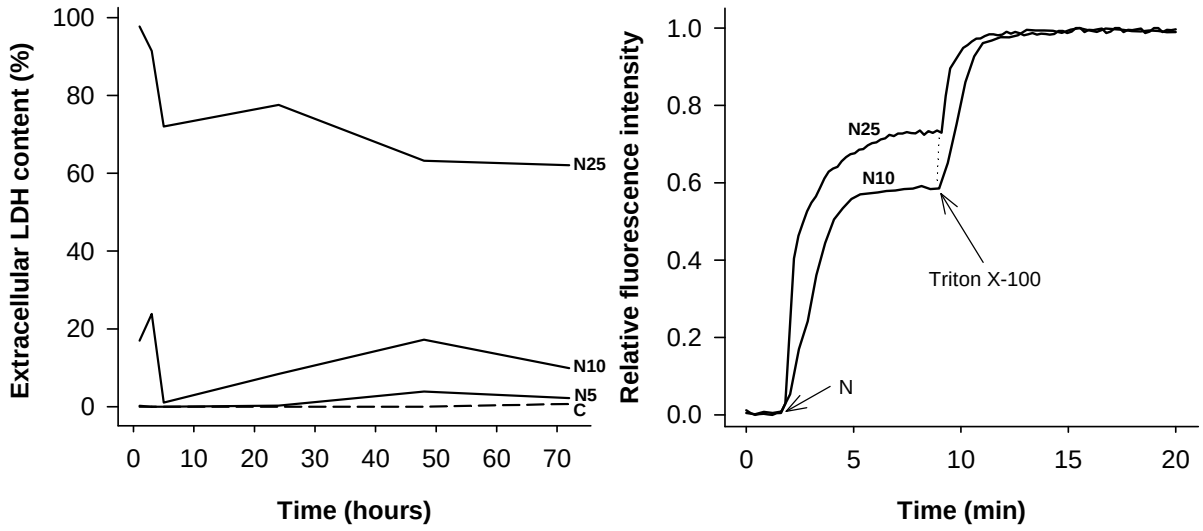


Figure 3.18. Left: Fluorescence measurement of extracellular LDH content with HeLa cells treated with muscotoxin.
Right: Fluorescence measurement of carboxyfluorescein leakage from LUVs.
Abbreviations stands for following: C = control nontreated cells, N = muscotoxin (numerical values in suffix reflect concentrations in μg/ml). Arrows indicate addition of denoted compounds.

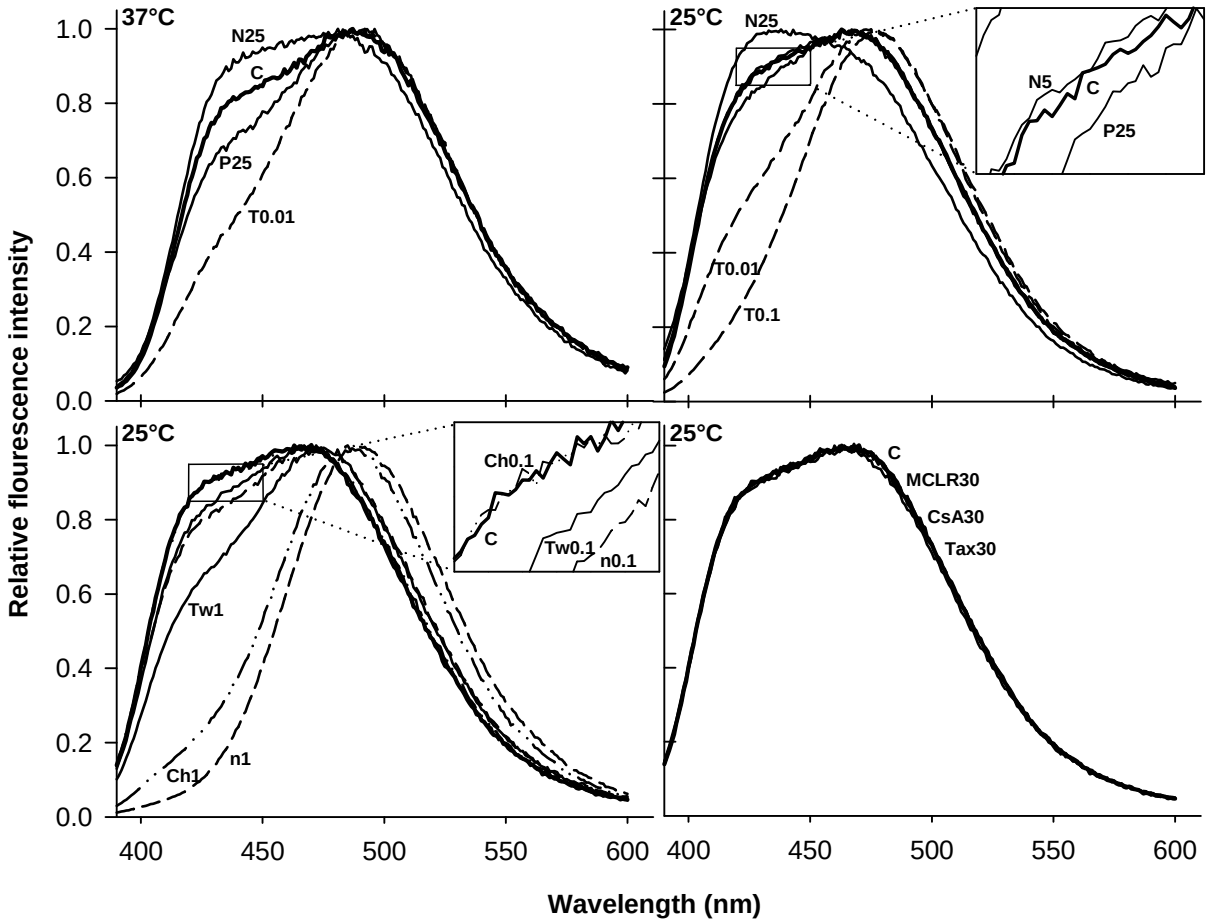


Figure 3.19. Top left: Fluorescence emmision spectra of Laurdan probe in 100nm LUVs at 37°C.
Top Right & Bottom: Fluorescence emmision spectra of Patman probe in 100nm LUVs at 25°C
Abbreviations stands for following: C = control nontreated LUVs, N = muscotoxin, P = Puwainaphycin F, MCLR = Microcystin-LR, CsA = Cyclosporin A, Tax = Taxol (numerical values in suffix reflect concentrations in μg/ml). T = Triton X-100, Tw = Tween 20, n = n-8-β-D-glukopyranosid, Ch = CHAPS (numerical values in suffix reflect concentrations in %). Rectangles show target magnified portion of plot.

The selfquenching experiment with carboxyfluorescein-filled LUVs (Large Unillamelar Vesicles) unearthed again valuable insight into mechanism of action (Fig. 3.18. Right). Rapid rise of permeabilizing effect at 25 μg/ml dose of muscotoxin as well as rather slower one at 10 μg/ml dose was congruent with preceding experiments but cell-free system displayed one siginificant difference. The lower concentration of cytotoxin was much more effective in permeabilization of LUVs than real cell membrane. Inducing 60% leak of carboxyfluorescein was nearly comparable to 70% value attained by higher concentration (25 μg/ml). Positive control in this experiment was 1% Triton X-100 which was proved to have 100% permeabilizing effect in our experimental settings.

As muscotoxin clearly interacts with the membrane independently of any other constituents and it is likewise primary insult responsible for toxic effect, only thing left to uncover was mode of interaction with phospholipid bilayer. LUVs interfused with variable domain-specific fluorescent probes were employed to provide insight into this matter.

Experimental LUVs composition was proposed according to composition of general mammalian plasmatic membrane striving to reflect its properties as much as possible.

Results obtained with synthetic bilayers provided the key contribution to final evaluation and interpretation of mechanism of action (Fig. 3.19). LUVs treated with lethal 25 μg/ml dose of muscotoxin induced significant decrease in mobility/viscosity of the dye´s microenvironment independently of temperature. Both Patman (localized at surface of membrane – 25°C) and Laurdan (localized rather deeper at hydrophilic heads – 37°C) registered similar changes, although microenvironment around Patman probe seemed to exhibit more intensive nonpolar shift (Fig. 3.19. Top right). Sublethal concentration (5 μg/ml) induced no observable effect at all.

Effect of 0.1 and 0.01% Triton X-100 exhibited opposite trend and probe microenvironment underwent extreme shift to higher wavelengths at both temperatures and probes. Same tendency was observed in treatment with other detergents (Fig. 3.19. Bottom left). Intensity of polar shifts were dependent on concentration and type of detergent but no compound was able to reduce the polarity of probe´s microenviroment.

Furthermore, to check whether the effect of muscotoxin is unique and not exhibited as an artifact (side effect) generally shared by peptides and other small natural drugs, the LUVs were treated with 30 μg/ml dose of cyclosporin A, taxol and microcystin-LR. All these compounds are known as membrane non-disrupters. Results were negative and fluorescence spectrum exhibited no shift in neither treatment (Fig. 3.19. Bottom right).

Finally, I have tested also cyanobacterial cytotoxin Puwainaphycin F, novel cyclic peptide recently isolated by my supervisor, that was found to possess similar membrane disrupting effect. Structurally, it is also a cyclic peptide although composition of amino acids differs and hybrid part is occupied by differently functionalized β-amino acid with longer aliphatic side chain. This peptide displayed effect rather congruent with results of low concentrated detergents, namely 0.1 % Tween 20, thus opposite to muscotoxin. Additionally, shift induced by Puwainaphycin F was more intensive at more fluid bilayers (37°C) than at rather rigid ones (25°C), again genuine opposite to effects induced by muscotoxin.

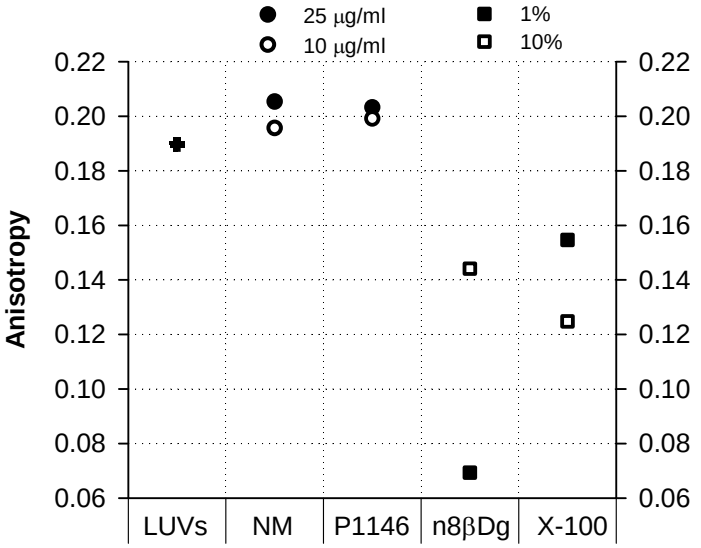


Figure 3.20. Steady-state anisotropy of TMA-DPH in LUVs measured at 25°C. LUVs = nontreated vesicles, NM = muscotoxin, P1146 = Puwainaphycin F, N8βDg = n-8-β-D-glukopyranosid, X-100 = Triton X-100.

Interaction of compounds with hydrophobic region of bilayers was measured by means of TMA-DPH anisotropy.

Both peptides induced slight increase in anisotropy (0.012 on

average) of DPH, reducing mobility of the probe´s microenvironment (Fig. 3.20). muscotoxin was somewhat more effective at higher comparable dose (25 μg/ml). Both tested detergents (Triton X-100 and n-8-β-D-glukopyranosid) displayed markedly opposite effect and decreased the anisotropy instead on average by 0.048 except for less concentrated 1% n-8-β-D-glukopyranosid which induced unexpectedly dramatic decrease to 0.12.

4. DISCUSSION

4.1. ANALYTIC CHEMISTRY

4.1.1. BIOASSAY-GUIDED FRACTIONATION

Although molecular ions *m/z* 885 and 1111 were tentatively identified as known cyanobacterial metabolites microginin 299-A (ISHIDA ET AL., 1997) and oscillapeptin G (SANO & KAYA, 1996), respectively, fragmentation analysis disproved this assignment. This demonstrates that comparing the molecular weight of unknown analytes with already described metabolites in literature is not a guarantee of realiable result and further structural analyses are needed.

4.1.2. AQUEOUS METHANOL IS THE MOST EFFICIENT EXTRACTION SOLVENT

Both MeOH and EtOH are strong solvents which likewise helped extraction process by dissolving cell membranes, and thus the high efficiency of extraction was expected. Importance of protic hydrogen for the extraction was well demonstrated by lower extraction power of MeCN, in which the substitution of hydroxy group by nitrile caused drastic decrease in efficiency. Pure water is indeed universal solvent and its efficiency was comparable to 50% MeOH suggesting that despite the molecule is consisted prevalently of nonpolar aminocids, resulting peptide is not completely nonpolar and thus partially dissolved in water.

Increasing the number of carbons in primary alcohol series lowered the efficiency of extraction confirming that polarity is one of the most critical factors for the extraction. Thus, totally nonpolar solvents like hexane and toluene displayed expected inefficacy muscotoxin extraction. Surprisingly, EtOAc displayed no extraction efficiency at all which is difficult result to explain. As a conclusion it is possible to say that muscotoxin extraction efficiency was most effective with solvents and mixtures of higher polarity and protic character, especially MeOH, which is routinely used in our laboratory as universal solvent.

4.1.3. THE MOST SUITABLE EXTRACT FOR PREPURIFICATION IS 50% MEOH PARTITIONED TWICE WITH ETOAC AND WATER IN RATIO 2:1:3

100% MeOH was immediately discarded from selection because the ratio of pigments/muscotoxin in extract was a lot higher than in diluted MeOH extracts (50-70%). From all potential extraction solvents (30-70% MeOH, 5% acetic acid, H_2O), 50% MeOH was selected as the most suitable solvent which retained extraction efficiency of MeOH and was the least

polluted by pigments. Although 5% acetic acid extract showed even less pigment content I´m convinced that such result is artifact caused by acid degradation pigments. Paradoxically, while water extracted appx. one half of muscotoxin compared to 100% MeOH it was more polluted by pigments.

Partitioning solvent with best characteristics was found to be EtOAc, routinely utilised by other researchers for similar works (TOSUN, PERSONAL COMMUNICATION; TERUYA ET AL., 2009A).General problem with partitioning was emulsion induced by excessive amount of extracted lipids and phosholipids but this was solved by lowering the concentration of extract, thus lipids. Furthermore, as the intensity of emulsification depends on temperature and partitiong process is exothermic, extract were freezed prior to partitioning process.

Generally, partitioning experiments demonstrated that optimal volume of partitioning soolvent (EtOAc) had to be equal to proportion of MeOH in extract. When the volume is lower tha optimal value, it was impossible to induce phase separation and in the opposite case (when the volume is higher) equlibrium of extracted compounds shifted towards EtOAc/MeOH phase making partitioning largely ineffective.

Eventually 50% MeOH was preferred to others due to two main reasons. Firstly, it displayed lowest level of emulsification and secondly, by introducing lower volume of EtOAc it allowed to process more extract in one funnel.

4.1.4. EFFECTIVE HPLC PURIFICATION REQUIRES TWO-STEP GRADIENT

MeOH/H_2O is considered as standard mobile phase for robust RP-HPLC separation methods of peptides and proteins and it does possess great potential when employed correctly (AGUILAR, 2004). Its potent solvating power allows it to dissolve complex natural mixtures otherwise difficultly soluble in other chromatographic solvents. Thus, this mobile phase was selected for the first separation step on reverse phase column.

Final gradient employed short interval in the beginning composed of equal proportions of both solvents which allowed more polar analytes to elute earlier. Additionally, it served as separation factor for related less polar analytes which would be otherwise inseparable by later changes in gradient. Following slow increase in portion of MeOH could afford better-resolved compounds of interest including muscotoxin.

Despite the chromatogram demonstrated adequate separation, this mobile phase composition had its flaws, mainly incomplete transparency of MeOH in absorption band of peptides (220nm). Moreover, the expected similarity of metabolites produced by studied strain make it a lot more difficult to obtain ideal chromatogram at this conditions and many compounds were poorly resolved from muscotoxin.

Obtaining first rough fraction of muscotoxin enabled to repurificate it by second step using other more chromatography-friendly solvents (MeCN in this case). Major advantages of this solvent are its complete transparency in UV, lowered backpressure in the system compared

with MeOH/H_2O and optimal separation abilities (TOSUN & TOMEK, 2010). Its unpleasant disadvantage is poor solvating ability. That is major drawback in all aplications which have to use another solvent to complement its deficiency (LINDNER & HELLIGER, 2004; YOSHIDA & OKADA, 1999).

In this case it was sorted out by decreasing MeCN content in the beginning of gradient and enhancing the solvating abilities of mobile phase by small contribution of MeOH to final ratio 9:1 (MeOH:MeCN). This amount of MeOH was moreover found to improve the separation abilities. Gradient was designed as a slow linear progression with constant part in the middle which could give time to resolve the analytes eluting together with muscotoxin, then followed by same slow linear trend as in previous MeOH/H_2O gradient up to the end of analysis.

Compared to MeOH/H_2O mobile phase, MeCN:MeOH/H_2O composition demonstrated much higher elution power and compounds were eluted at lower MeCN:MeOH proportions in gradient although generally, MeCN has lower elution strength than MeOH. During separation with MeOH/H_2O, concentration of MeOH had to be at least 70% to assure elution of muscotoxin in reasonable time as opposed to separation with MeCN:MeOH/H_2O mobile phase where the proportion of MeCN:MeOH never exceeded 25%. Behaviour of individual components in column during separation is complex matter but this result suggests that analyte-solvent interactions in the case of MeCN:MeOH mobile phase was much stronger.

Furthermore, no change in preparative chromatographic condition didn´t resolve the congeners of muscotoxin, possibly due to high concentration of analytes or inferior characteristics of Reprosil sorbent compared to Zorbax used in our analytical column.

4.1.5. THREE FRACTIONS SHARE SIMILAR ELUTION AND FRAGMENTATION PATTERN

HPLC-MS analysis of obtained fractions revealed that *Nostoc muscorum* strain synthesized at least three compounds with expected similar scaffold, which was deduced based on the similarities in their MS/MS spectra. In the case of muscotoxin (*m/z* 1212) and its congener (*m/z* 1226) it was proposed that the structures differ only in minor detail and this hypothesis was confirmed by NMR and MS fragmentation analyses. Difference was indeed only in γ-methylated Pro subunit (*m/z* +14) as discussed in next section. Due to the substrate flexibility of NRPS/PKS biosynthetic enzymes (ROHR, 2006), this alternative is feasible and many congeners of another cyanobacterial peptides are proposed to be synthesized in this way (BONNARD ET AL. 2007). Although it is difficult to suggest the difference in *m/z* 1112 structure, major fragmented ions were identical to other two structures although the spectrum missed the water losses. That could be caused by Ser/Pro substitution, modification or complete loss of β-amino acid. All these suggestions are clearly hypothetical and only additional structural analysis will reveal the genuine structure.

If both of these structures (*m/z* 1112 and muscotoxin) will be proved to share similar scaffold it allows us to identify the pharmacophore in muscotoxin because *m/z* 1112 exhibited no

cytotoxic effect. Collectively, that result would allow us to propose SAR and biosynthetic pathways of peptides present in this strain. Unfortunately, as the *m/z* 1226 was purified serendipitously only few weeks ago before finishing the thesis and in small quantity (appx. 0.8 mg) I didn´t manage to test its bioactivity and instead it was subjected to structural analysis.

Due to the same difference (*m/z* +14) in molecular weights and elution patterns (data not shown) of metabolites *m/z* 885 and 899 (Fig. 3.1) I suspect that all substances synthesized by this strain will carry similar scaffold. Moreover, *m/z* 885 was proved to be toxic to brine shrimps although lacking cytotoxic activity (HISEM ET AL., 2010).

On the other hand, much more confusing was the identical elution of compounds´ congeners/conformers. Since these compounds exhibited identical molecular mass, firstly I though that these were structural isomers of the main compound. However, it was rejected by NMR analysis. Moreover, injection of diluted sample disproved misinterpretation caused by high concentration of analytes. Although NMR experiments ruled out presence of conformers I´m not aware how to explain the impossibility in separation of these structural entities/conformers than that all substances exist in some defined ratio of its constituents in the solution and after separation they maintain the same original equilibrium shifting among its forms. More specific study will be needed to confirm such theory.

4.1.6. CYTOTOXIN IS CYCLIC HYBRID DEPSIPEPTIDE OF UNIQUE STRUCTURE WITH SUMMARY FORMULA $C_{58}H_{90}N_{12}O_{16}$ AND MOLECULAR WEIGHT 1210.6666 DA

As an undecapeptide, muscotoxin belongs to a scarce group of cyanobacterial metabolites with more than 10 subunits in the cycle. Moreover, it carries two hybrid subunits probably derived from PKS biosynthetic route. Unique β-amino acid (Adhde) and glyceric acid which is incorporated into ring via depsipeptide bond by esterification of carboxyl present on Dhb amino acid. To my knowledge, no cyclic depsipeptide with β-amino acid has been found to possess ester bond at other place than at β-amino acid hydroxy group. Thus, I herein demonstrate this unique feature in hybrid β-amino acid containing peptides.

Additionaly, molecule contains nonproteinogenic Dhb, relatively infrequent isoforms D-Gln, D-*allo*-Ile, and its congener was described to possess rare γ-MePro. Apart from these exceptions, the rest of the peptidic backbone is comprised of standard amino acids, probably in L-forms. (chirality of Pro, glyceric acid and Adhde subunits was not elucidated to date of finishing this thesis). Despite all amino acids in the rest of the cycle are connected via simple amide bond, glyceric acid incorporation represents interesting chemotype enriching the biodiversity pool of cyclic peptides. This demonstrates that the structural variability of cyanobacterial metabolites has still much to offer.

Only about 20 β-amino acid contataining peptides with 10-14 subunits in cycle were described so far. Cyclic decapeptides like puwainaphycins A-E (GREGSON ET AL., 1992), F-G (HROUZEK ET AL., 2010), calophycin (MOON ET AL., 1992) or largamide H (PLAZA & BEWLEY, 2006), and

undecapeptides lobocyclamide A (MACMILLAN ET AL., 2002), apart from stereochemistry of Dhb subunit identical hormothamnin A and laxaphycin A (GERWICK ET AL., 1992; BONNARD ET AL., 2007), schizotrin A (PERGAMENT & CARMELI, 1994), scytonemin A (HELMS ET AL., 1988), pahayokolides (AN ET AL., 2007) and recently identified lyngbyazothrins (ZAINUDDIN ET AL., 2009). Dodecapeptides are even more rare represented only by laxaphycin B (BONNARD ET AL., 2007) or lobocyclamide B (MACMILLAN ET AL., 2002). Higher amount of subunits (13-14) were found only in two congeners of malevamides B and C (HORGEN ET AL., 2000).

All these structures contain single β-amino acid connected to the ring via double amide linkages except from malevamides which integrates β-amino acid (C_6-C_8, 2-methylacyl-) via esterification of hydroxy group on the ring. 3-amino group forms amide bond as it is typical for other structures of this type.

β-amino acids of all other mentioned compounds differ by functionalizations on the chain and its length. Puwainaphycins and calophycin display longest aliphatic chains (C_{14} - C_{18}) substituted at 2- and 4- position by hydroxyl and methyl, respectively. In some of the puwainaphycines there are also oxo- functionalizations and chlorine atom present. Lobocyclamides, laxaphycines and hormothamnin A contain shorter β-amino acid side chains (C_8 - C_{10}) completely devoid of any functionalizations, let alone little similarities with muscotoxin present on the ring.

Compounds that partially reflects the structure of muscotoxin were found to be schizotrin A, pahayokolides, lyngbyazothrins and largamide H. Furthemore, except for largamide H, all are likewise undecapeptides. Largamide H contains identically functionalized β-amino acid (2,5-dihydroxy-3-aminoacyl-) although it is one acetyl unit longer (C_{12}) than in structure of muscotoxin (C_{10}). But the rest of its molecule doesn´t possess much similarities with muscotoxin. Conversely, other three candidates (pahayokolidkes, lyngbyazothrins and schizothrin) demonstrate close similarity in the ring, but β-amino acid is more heavily functionalized (3-amino-2,5,7,8-tetrahydroxy-10-methyl-decanoic acid). Additionally, it has esterified C_5 hydroxy group by various modified amino acids (Fig. 4.1).

All four structures, including isolated muscotoxin, contain identical partial sequence defined by two prolines on the ends (Pro-[β-amino acid]-Gln-Gly-Pro). The second part of the ring bears similarity in distribution of polar and nonpolar subunits. The quinary sequence contains Dhb in the center and Thr/Ser/glyceric next to it, enclosed by various nonpolar aminoacids (NH_2-Nonpolar-Thr/Ser-Dhb-Thr/Ser/glyceric acid-Nonpolar-COOH). Despite the nonpolar constituents at N-terminus of ring sequence are more variable (Dhb/Ile/Val), the C-terminus is occupied by Ile in muscotoxin as opposed to only HomoPhe/Tyr in schizothrin, pahayokolides and lyngbyazothrins.

Conclusively, β-amino acid of muscotoxin shows the best similarity with largamide H and ring is most closely reflected by schizothrin A and pahayakolide B which both don´t contain glyceric acid, depsipeptide bond, and nonpolar termini in described quinary sequence are substituted by Ile in structure of muscotoxin. Other than, the remaining sequence of the ring is identical (Fig. 4.1).

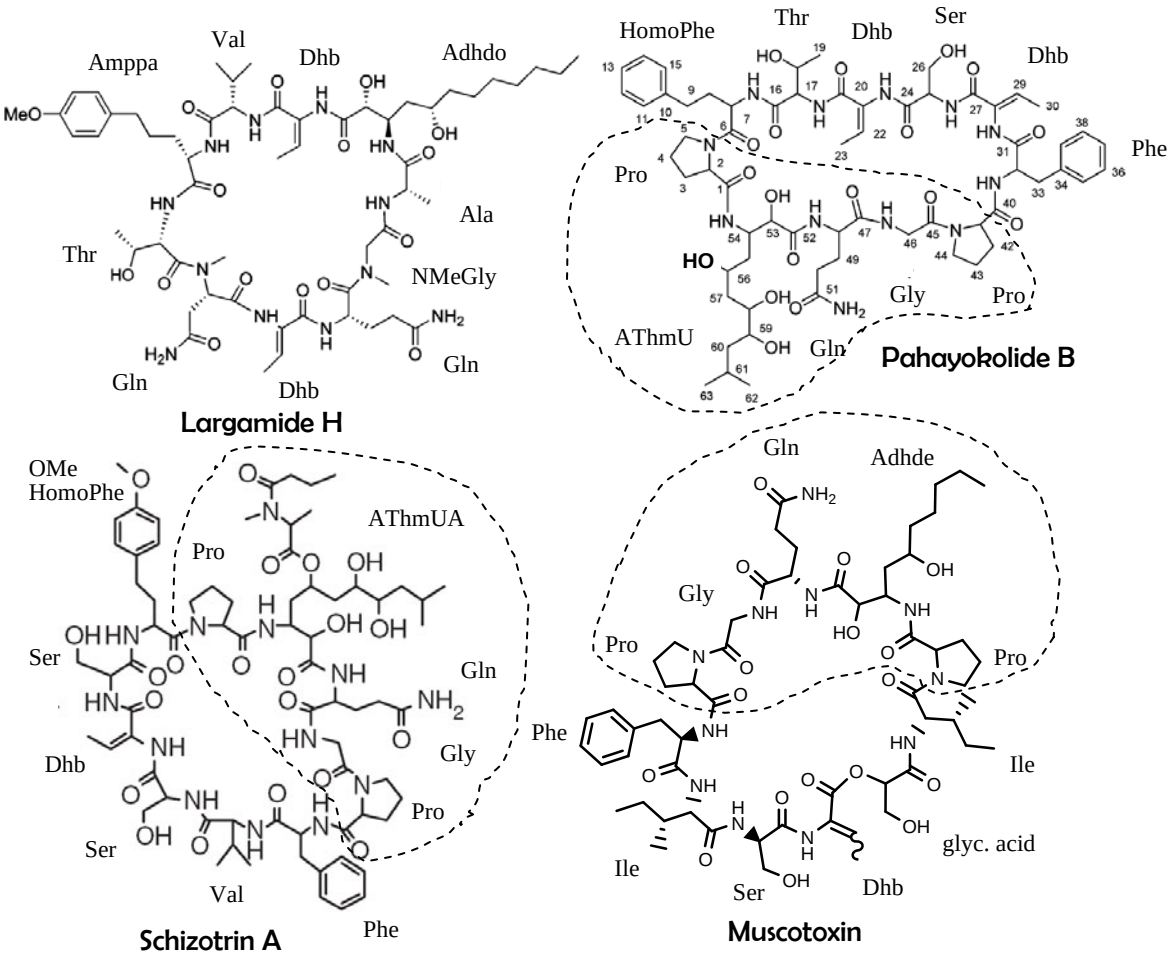


Figure 4.1. Structures of four similar β-amino acid cyclic peptides with denoted structural subunits. Dashed line indicates identical quinary sequence in all metabolites

4.2. CELL TOXICOLOGY AND MECHANISM OF ACTION

4.2.1. MUSCOTOXIN IS MODERATELY LOW POTENT WITH MINOR SELECTIVITY FOR ADHERENT CELL LINE AND BEHAVES IN DOSE-DEPENDENT MANNER SIMILARLY TO DETERGENT TRITON X-100

IC_{50} value 12 μg/ml towards HeLa cell line is quite high in comparison with other cyanobacterial cytotoxins (Review - Table 1.1, Fig. 1.11) and muscotoxin thus belongs to the group of the least efficient cyanobacterial cytotoxins described in the literature. Even this fact alone suggests that the potential of this compound as antitumour drug is low. Also, the mechanism of action suggests that it will act in a rather non-selective manner. Likewise its structural features predetermine it to such low potency because so far, no cyanobacterial metabolite with 10 and more subunits containing aliphatic β-amino acid was described as antitumor agent.

Higher toxicity to HeLa cell line is most likely caused by greater surface area rendering them more susceptible to membrane injury than small nonadherent lymphocytes. Additionally, cell lines originate from different tissues which could possibly play some role in the effect, despite its general nonspecifity.

It was proved that cytotoxic effect behaves in a dose-dependent manner. At two highest tested doses (20-30 μg/ml) the effect results probably in rapid necrotic cell death, although with lower concentration (20 μg/ml) its onset is postponed for 15 hours. Nonetheless, the damage is probably so substantial that cell cannot reverse the fate. Opposite trend is observed at lower concentrations where muscotoxin seems to only reduce the overall fitness of the cells (metabolic activity) and eventually the cell (or subpopulation) is able to survive. Cytotoxin probably induces some pathological changes that can be reversed or affect only specific subpopulations (e.g. during or after cell division, weaker individuals etc.).

The same trend was observed with detergent Triton X-100 at doses which induced comparable morphological alterations. These results suggest that the toxin could possibly solubilize the cellular membranes as Triton did.

4.2.2. MUSCOTOXIN INDUCES MEMBRANE INJURY, CHROMATIN CONDENSATION; SWELLING AND FRAGMENTATION OF MITOCHONDRIA

Up to this time, mechanism of action of Triton X-100 was considered nearly identical to that of muscotoxin and indeed even fluorescent staining of nuclei and actin cytoskeleton in treated cells didn´t reveal major differences in their phenotypes. But first difference were found when observing the mitochondria. Both compounds caused mitochondrial disruption, however, in different way. At least the phenotype of mitochondria after treatment by muscotoxin and Triton-X were completely different. Muscotoxin induced swelling and rounding of mitochondria which resulted in uncoupling and fragmentation judging by the lowered intensity of potential-sensitive MitoTracker dye. Such a phenomenon is attributed to the increase of $[Ca^{2+}]i$ (BOUSTANY ET AL., 2002), thus muscotoxin probably didn´t explicitly interact with mitochondria themselves. As the alterations of mitochondria are frequently associated with induction of cell death (CHEN ET AL., 2001) this was a clear sign of cell decease induced by perturbed membrane and subsequent leakage of ions, altering the pH and generally violating the cell homeostasis.

On the other hand, Triton X-100 completely dissolved and disintegrated mitochondria. Based on this finding, I´m suggesting that cytotoxin is not actually acting like a detergent and probably don´t even enter the cytosol. Mitochondrial damage is only one of the phenotypes induced by interaction of muscotoxin with plasmatic membrane.

Furthermore, Triton X-100 washed away the cells from growth surface at IC_{100} dose (0.05% - 900 μM) as opposed to muscotoxin treatment where at IC_{100} (25 μg/ml – 20 μM) the cells were still attached to the surface confirming that mechanism of action is different to

detergent. The effect of muscotoxin is nonspecific but still about 36x more effective than Triton X-100.

The *in vivo* observation showed that membrane damage is forsooth the primary insult which intensity is dose-dependent as confirmed in alamarBlue experiments. When low concentration was applied, the cell demonstrated nearly no morphological alterations but mitochondria still suffered damage which correlates well with previous experiments and proves that even if low concentration of toxin induces pathological effect, the fate of the cell can be reversed (TRUMP & BEREZESKY, 1996) and doesn´t result in cell death, only reduce its fitness. Toxin is apparently able to perturb the membrane integrity even at sublethal concentrations as was proved by membrane permeability experiments that will be discussed next.

4.2.3. MEMBRANE BECOMES RAPIDLY PERMEABLE FOR IONS, SMALL COMPOUNDS AND MACROMOLECULES. MUSCOTOXIN DISPLAYS SPECIFITY FOR PHOSHOLIPIDS AND ACTS INDEPENDENTLY OF OTHER MEMBRANE CONSTITUENTS.

Measurements of $[Ca^{2+}]i$ and LDH leakage confirmed that effect is rapid. Furthermore, judging by the decreases in $[Ca^{2+}]i$ and LDH concentrations after initial insult, I suggest that cells can possibly react to the insult in swift manner pumping out the leaked ions, buffering the change in pH and if the damage is not excessive, they are even able to repair the damaged bilayer and survive (ČERNÝ, PERSONAL COMMUNICATION).

Treatment with ionomycin (selective calcium ionophore) confirmed this hypothesis. As the ionomycin is selective and didn´t induce any physical damage to the membrane compared to muscotoxin, it is possible to suggest that observed decreases in concentrations correlates really to the cells fighting off the increased level of ions. Firstly, it was suggested it was artifact in FURA measurement because at such high concentrations of $[Ca^{2+}]i$ the experiment is unreliable.

Observing the effect of muscotoxin on synthetic LUVs filled with carboxyfluorescein revealed that cell-free system indeed lack the response of living cells. 70% release of carboxyfluorescein at 25 μg/ml dose of muscotoxin is in accord with LDH experiments where 80% value was observed. But 60% value after treatment with 10 μg/ml didn´t prove the dose-dependency observed in other treatments with living cells, rather opposite. It is possible that synthetic bilayers are more susceptible to the effect of muscotoxin but lack of response to the membrane damage observed in living cells is more feasible explanation in that case. Furthermore, this experiment proved that muscotoxin acts independently of any other cellular membrane consituents, thus it is phospholipid-specific.

The ability of cells to „heal“ themselves was additionally proved by microscopic observation of treated cells in time (data not shown). If the HeLa cells were treated with IC_{50} dose of muscotoxin, after 1 h typical phenotypes of plasmatic membrane and mitochondria were observed. But as the concentration was not lethal, the majority of cells were still living as proved

by active endocytosis. The small fractions of cells were dead. If the same living cells were observed after 24 hours of treatment they displayed morphological abberations and abberant structure of mitochondria but they survived the tratment with muscotoxin.

4.2.4. MUSCOTOXIN HAS HIGH AFFINITY FOR PLAIN PHOSPHOLIPID MEMBRANE AND INTERACTS WITH BILAYER AT BOTH HYDROPHOBIC AND HYDROPHILIC DOMAIN. INTERACTION IS PROBABLY OF NOVEL NATURE DIAMETRICALLY OPPOSITE TO THE EFFECT OF DETERGENTS AND SIMILAR PEPTIDE.

At IC_{100} dose for HeLa cells (25 μg/ml) muscotoxin induced significant decrease in polarity of dye´s microenvironment at hydrophilic region of bilayer. As opposed to the tratments with detergents where the trend was inverse and the microenvironment polarity increased. This can be considered as a proof that muscotoxin is not dissolving phosholipids because membrane didnt become hydrated as it was in the case of detergents.

Judging by the decrease of polarity about surface-located probe it seems that muscotoxin makes the bilayer even more compact, thus less fluid. But how is that connected with so potent membrane permeabilization of cytotoxin? Apparently, the more rigid conformation of bilayer might make it less resistant physically, thus more susceptible for breaks and tears than fluid one which is very "elastic". Thus, two possible explanations are proposed and core principle of both is extremely high affinity of muscotoxin for phosholipids. Cytotoxin is probably left interfused in the membrane even after successful permeabilization. Moreover, we suggest that β-amino acid will play significant role in the observed bioactivity although largamide H, which contains identically functionalized β-amino acid (only one acetyl unit shorter) didn´t exhibit cytotoxic activity (PLAZA & BEWLEY, 2006). Thus, possibly only the specific layout of all subunits including β-amino acid length and functionalization will induce cytotoxic effect as suggested by Gerwick´s and Bonnard´s group. They proposed that distribution of hydrophilic/hydrophobic subunits in the structure is crucial for its bioactivity (GERWICK ETAL., 1992; BONNARD ET AL., 2007).

First idea is based on condition that rigidized membrane becomes less resistant physically and can be easily shattered as the thin block of ice. Thus, when the muscotoxin binds on the surface domain of bilayer, it rigidizes membrane conformation by interfusing inside which results in random cracks and tears compromising the membrane integrity. Furthermore, higher effectivity of muscotoxin at lower temperature strenghten this idea.

The second idea stems from the assumption that nonpolar shift is induced by tightening (aggregation) of phospholipid domains around muscotoxin. Thus, after muscotoxin binds to membrane, this binding induces conformation change in the molecule and induce tiny gap in the membrane. As a response, phospholipids will start to migrate in this place to "fill" the gap. This migration could tear the most vulnerable places on the membrane e.g. sections without the less

intermembrane proteins or marginal parts of the cell. It is difficult to interpret such unprecedented result and these ideas are incertain.

On the other hand, it is certain that cytotoxin interacts with membrane at hydrophilic domain (polar heads). Although some interaction was recorded even at hydrophobic part of bilayer. After the treatment of LUVs with both peptides (muscotoxin and puwainaphycin F at 25 μg/ml) slight shift in anisotropy of TMA-DPH was observed meaning that there is some interaction even at hydrophobic domain of bilayer but comparing these results to detergents it was found negligible and moreover in opposite direction. Detergents made the hydrophobic region of bilayer more polar, thus clearly dissolving it unlike the peptides. Furthermore, the small rise in anisotropy induced by peptides can be assigned to interaction with the probe itself restricting its motional freedom.

The membrane interaction observed in treatment with muscotoxin was proved to be exclusive. Such membrane rigidizing effect wasn´t observed neither by any other compound tested in the experiment (puwainaphycin F, detergents, taxol, microcystin-LR and cyclosporine A) and only somocystinamide A was found to exhibit interaction with membrane. But this interaction is of completely different nature and induces apoptosis instead of necrosis (WRASIDLO ET AL., 2008).

Additionally, the large peptides (10-14 subunits) with β–amino acid, only laxaphycins were found to be cytotoxic but authors didn´t mention its mechanism of action, so I could´t make any comparison (BONNARD ET AL., 2007; FRANKMOLLE ET AL., 1992).

Although such effect wasn´t described by any other researchers it doesn´t mean that similar compounds like schizothrin A, pahayakolides, lyngbyazothrins or largamide H don´t exhibit it. It is highly probable that its inventors only didn´t test their compounds for such interaction. Maybe testing the mentioned compounds along with muscotoxin in membrane permeabilization experiments would reveal possible connection.

It is apparent that such effects are not interesting for pharmacological purposes, thus research is not interested in elucidating of the mechanism of action of necrosis-inducing compounds. On the other hand, many large cyclopeptides containing β-amino acids were described as antifungal and antibiotic agents (MACMILLAN ET AL., 2002; GERWICK ET AL., 1992; PERGAMENT & CARMELI, 1994). And possibly such bioactivity doesn´t need its mechanism of action described.

Further studies will hopefully reveal the true nature of this puzzling activity. But as for now the mechanism of action of cell death induction is considered as novel among cyanobacterial cytotoxins.

5. CONCLUSION

- Two novel cyclic undecapeptides muscotoxins have been isolated from terrestrial cyanobacteria *Nostoc muscorum*
- Structure features new β-amino acid Adhde (2-amino-3,5-dihydroxydecanoic acid) and glyceric acid which is incorporated in to the ring via depsipeptide bond
- Peptide is a mildly active cytotoxin with IC_{50} 12 μg/ml on HeLa cancer cell line
- It induces dose-dependent necrotic cell death by rapid permeabilization of the plasmatic membrane via unknown interaction
- Mechanism of action is probably of novel nature characterized by high affinity of peptide for bilayer phosholipids surface and subsequent membrane rigidization
- Antitumour application of this compound is not feasible

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