

University of South Bohemia in České Budějovice

Faculty of Science

The evaluation of the photochemical activity of cyanochelin 1026 and its photolytic fragments

Master thesis

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České Budějovice, 2022

Jiřík F. (2022): The evaluation of the photochemical activity of cyanochelin 1026 and its photolytic fragments. Mgr. Thesis, in English. – 104 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Anotace:

A cyanobacterial siderophore cyanochelin 1026 is capable to coordinate Fe^{3+} cation into stable complexes, which facilitates the reduction of the coordinated iron atom upon photodegradation of the compound. The kinetics of photodegradation of cyanochelin 1026 was studied upon the exposition of the cyanochelin 1026- Fe^{3+} complex to UV light. The photoproducts of photolysis were identified and studied for further photoreduction potential towards Fe^{3+} cations.

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Acknowledgment

I would like to express my gratitude to Mgr. Tomáš Galica PhD. and RNDr. Pavel Hrouzek PhD. for their introduction to the field of cyanobacterial lipopeptides, to valuable practical advice and support during my work.

I would like to show my gratitude to Mgr. Jan Hájek PhD and Dr. Kumar Saurav, Ph.D. for a practical introduction to HPLC-DAD and HPLC-MS/MS analytical methods. My gratitude also belongs to all staff of the Centrum ALGATECH for their very kind attitude and willingness to help whenever I asked for.

I would like to thank the Dr. Valeria Costantino PhD. from “TheBlueChemistryLab”, Department of Pharmacy, University of Naples Federico II, Napoli, Italy for structural analysis of cyanochelin 1026 with NMR method.

This thesis was done at Institute of Microbiology, CAS in laboratories of the Centrum ALGATECH. This work was financially supported Czech Science Foundation GACR project: ‘Iron monopolization versus community service: the two faces of cyanobacterial beta-hydroxy aspartate lipopeptides (grant No. 22-05478S, investigator: RNDr. Pavel Hrouzek PhD., Centre Algatech, Institute of Microbiology, The Czech Academy of Sciences (CAS), Třeboň, Czech Republic).

Abbreviations

ACN- acetonitrile

ATP-adenosine triphosphate

BPDS- bathophenanthroline disulfonic acid

DAD- Diode Array Detection

Dark- no light exposition

dH₂O- destiled water

ESI- electrospray ionization

EtAc- ethyl acetate

FA-formic acid

HPLC- high performance liquid chromatography

MeOH- methanol

MS/MS- tandem mass spectrometry

MOPS- 3-(N-Morpholino)propane sulfonic acid

NRPS-non-ribosomal peptide synthase

PFP- pentafluorophenyl

PKS- polyketide synthase

PS- photosynthetic apparatus

qTOF- quadrupole time of flight mass spectrometer

TFA- trifluoroacetic acid

UV- light in near ultraviolet spectra

Vis- light in visible spectra

1. Abstract

Cyanobacteria represent an important component of many ecosystems in the world, which play various roles in biological systems as well as in biogeochemical cycling of elements. The production of secondary metabolites is a frequent process found in most of the cyanobacteria. The secondary metabolites are characterized as low molecular mass compounds, which are produced through special metabolic pathways. A function of these compounds is to mediate the abiotic and biotic interactions of the organism with the environment for the purpose to achieve a selective life advantage (Gokulan et al., 2014). Lipopeptides represent a significant group of cyanobacterial secondary metabolites including. These compounds are produced through combination of two metabolic pathways: non-ribosomal peptide synthase (NRPS) and polyketide synthase (PKS) (Mareš et al., 2014). The molecules of lipopeptides consist of lipidic tail from modified fatty acids (PKS) and peptide chain with length from one to dozens of amino acids (NRPS) (Cochrane et al., 2016). Most of the lipopeptides exhibit some type of bioactivity, which are utilized in many life-enhancing strategies, e.g. acquisition of nutrients through siderophores (Sandy et al., 2009).

Iron is a fundamental trace metal required for maintenance of basal cell function. Despite iron forms 32% of Earth mass, most of the iron on the Earth crust is present in biologically unavailable forms (J. W. Morgan et al., 1980). Therefore, the prokaryotic organisms have evolved many strategies of iron acquisition including siderophore-mediated acquisition. Siderophores are natural organic compounds with a high affinity towards iron (III), which are produced in iron deficiency (Kranzler et al., 2013). Some siderophores can facilitate the photoreduction of iron (III) to iron (II) upon self-degradation. The photoreduction potential has been reported in several cyanobacterial lipopeptides containing β -hydroxy aspartic acid in their peptide chain (Hardy et al., 2019). The cyanochelin 1026 is a newly discovered β -hydroxy aspartic acid-containing lipopeptide, which is produced by cyanobacterium *Leptolyngbya* sp. NIES 3755 (Galica et al., 2021). The photoreduction potential of cyanochelin 1026 to iron (III) was revealed recently (Galica et al., 2021), however, so far there is no information about the reaction mechanism as well as about the photoproducts of the cyanochelin 1026 photodegradation.

2. Theoretical background

2.1 Cyanobacteria and their role in ecosystems

2.1.1. Characterization of cyanobacteria

Cyanobacteria are gram-negative bacteria with the capability to perform oxygenic photosynthesis. This group is known to be very diverse in many aspects of life including morphology, metabolism, life strategies, habitat, etc. Cyanobacteria live in majority of the terrestrial and aquatic habitats including oceans, fresh waters, deserts, soils, and rocks. Besides the mild environmental conditions, cyanobacteria also grow in extreme conditions of arctic regions, hot springs, hypersaline waters, or other extreme habitats (Whitton, 2012).

The cell of cyanobacteria is in many features a common prokaryotic cell, but there are several characteristic traits as well. The cyanobacterial cell is gram-negative with an inner cytoplasmic membrane and an outer bacterial membrane, which fit in a thin peptidoglycan cell wall. In the cytosol there is a circular DNA surrounded by free floating ribosomes. As in the other prokaryotes, there are no organelles or vacuoles in the cell, however, membrane embedded structures responsible for photosynthesis (thylakoids) or gas vesicles are frequently found in cyanobacterial cells (Anagnostidis et al., 1990). The size of the cell, however, differs significantly between cyanobacteria and other prokaryotic cells. The size of most bacteria ranges usually from 0,1 to 10 μm (Hartman et al., 1993), while the cell diameter of cyanobacteria may be significantly higher with a diameter from 1 μm up to 50 μm (Sánchez-Baracaldo, 2015). Although the cell size is an obvious and easy recognizing feature of the cyanobacterial cell, the most significant difference between cyanobacteria and other prokaryotes is represented by the photosynthetic (PS) apparatus. There are many autotrophic organisms in the prokaryotic world, but only the cyanobacterial PS apparatus can utilize water as a source of electrons for generation of proton gradient finally used in ATP synthesis, which is recognized as a unique trait for this group of organisms (Sánchez-Baracaldo et al., 2020). The photosynthetic apparatus is anchored to the thylakoid membranes, which are compartments on cytoplasmic membrane arranged in a manner specific for each cyanobacterial group. The presence of these membrane structures represents also another feature typical for the cyanobacterial cell (Komárek et al., 2014). The cyanobacteria stand at the beginning of the evolution of recent phototrophic organisms including higher eukaryotic phototrophs. In the process of endosymbiosis the

cyanobacterial cells were engulfed by bigger heterotrophic cells and gradually became chloroplasts (Moore et al., 2019).

The morphology of cyanobacteria is relatively variable, and cyanobacteria can adopt various life-strategies. They can live as single-cell entities with no connections to the other cells of the same species in the surrounding. Marine cyanobacteria such as *Synechococcus* represent an example of typical unicellular cyanobacterium (Kaneko et al., 1997). A special lifestyle is observed in the case of colonial cyanobacteria. The main trait of colonial cyanobacteria is the formation of colonies counting from tens to hundreds of unicellular individuals. The physical connection between the cells is often provided through slime cover, which is produced and shared by members of the colony. Although the cyanobacteria in these colonies collaborate tightly with each other, there have been no closer cell connections or cell differentiation within the colonies observed. An example of such behaviour represents the fresh-water cyanobacteria genus *Chroococcus* (Anagnostidis et al., 1990). The second group of cyanobacteria comprises filamentous species where individual cells are communicating via intracellular connections. The cells within filaments may exhibit cell differentiation (heterocysts, akinetes, hormogonia, etc.), which provides the cyanobacterium variety of life advantages. In some of the filamentous cyanobacteria the formation of more complex macroscopic colonies is observed. A genus *Leptolyngbya*, including the model organism of this work *Leptolyngbya* sp. NIES 3755 (Fig.1) belongs among unbranched filamentous cyanobacteria with no cell differentiation observed (Komárek et al., 2015).

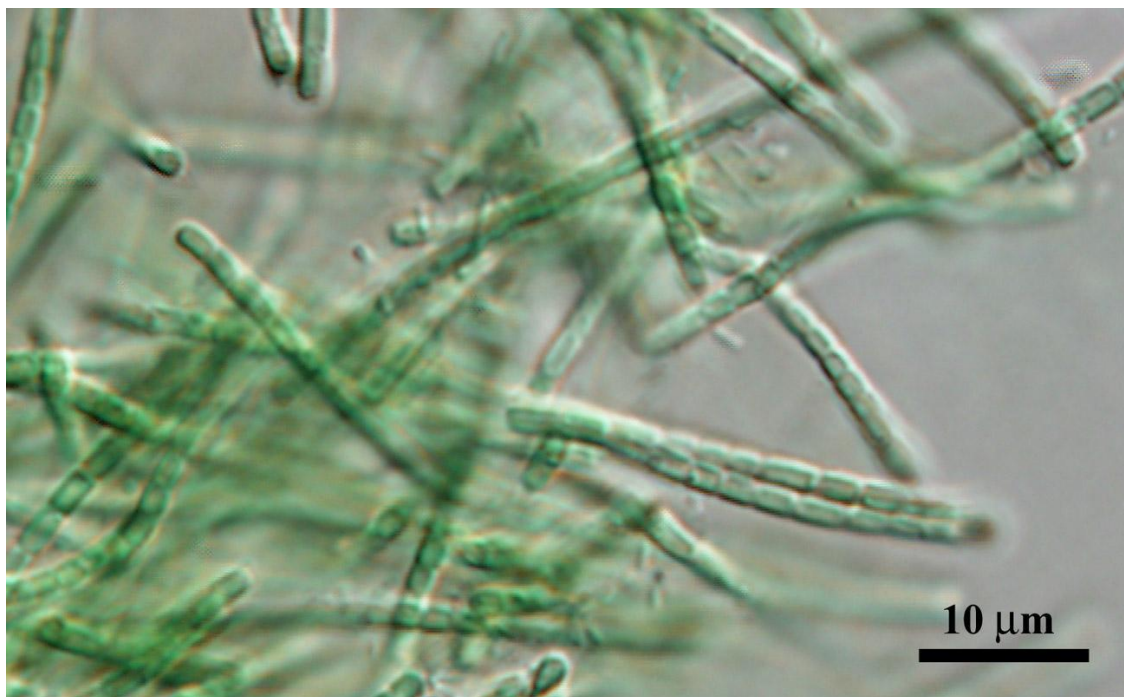


Fig.1.: Filamentous non-heterocystic cyanobacterium *Leptolyngbya* sp. (*Leptolyngbya* Sp. | CCALA, n.d.)

2.1.2.Role in ecosystems-water and soil

As mentioned in the previous chapter (2.1.1) cyanobacteria are versatile organisms that can inhabit many aquatic and terrestrial ecosystems. Their function in the ecosystems is very diverse depending on the lifestyle of the cyanobacteria as well as on system the cyanobacteria are accommodated in. Cyanobacteria represent a fundamental component formajority of aquatic ecosystems, which plays a crucial role in global carbon, nitrogen, and oxygen cycling. Due to their abundance, aquatic and especially ocean cyanobacteria contribute significantly to the production of atmospheric oxygen and reduction of carbon dioxide concentrations in the atmosphere. Among the most abundant cyanobacteria responsible for oxygen production belongs unicellular *Prochlorococcus*, widespread marinecyanobacterium omnipresent in of the oceans in the world(Grimes, 2009). In soilthe cyanobacteria can also help to improve soil quality and water retention and reduce soil erosion due to the formation of various polysaccharides, which stick soil particles together and thus prevent their displacement (Belnap et al., 1993).

Besides involvement in carbon and oxygen cycling cyanobacteria contribute also to nitrogen cycling in the oceans. This function is provided through the ability of some cyanobacteria to fix atmospheric N_2 and convert it to water-soluble forms, which are

accessible to other organisms. Nitrogen fixation is feasible due to the unique enzyme system called nitrogenase. The nitrogenase itself consists of two subunits, both containing Fe cations in the active centre, which serve as catalysts to the N_2 reduction reaction (Fay, 1992). Fixation of N_2 is from the ecological point of view an important process, which helps to balance the loss of nitrogen from water and soil usually associated with bacteria decomposition of biomass (Kuhlbusch et al., 1991).

Despite the presence of cyanobacteria in aquatic and soil ecosystems is usually beneficial, their populations might become dangerous to the surrounding organisms when their growth exceeds acceptable levels. The extensive growth of cyanobacteria results in formation of cyanobacterial blooms (Mullineaux et al., 2021). These might cover significant areas of water surface with a layer of biomass the light cannot penetrate through (Fig.2). As a consequence, the organisms below the bloom layer suffer from light depletion subsequently accompanied by oxygen depletion due to respiration of the blooming biomass in dark phase of the day, which becomes lethal for a majority of aquatic organisms (Mullineaux et al., 2021).



*Fig.2.: The water surface of Lake Chaohu (China) covered by dense layer (bloom) of *Microcystis aeruginosa* biomass (Chen et al., 2016)*

Cyanobacterial toxins represent another danger related to the blooms. The metabolism of cyanobacteria includes many metabolic side pathways, which produce various organic compounds with bioactive properties (see chapter 2.2 for details) (Huang et al.,

2019). The bioactive compounds are thought to enhance cyanobacterial fitness via improvement nutrient acquisition, mitigation of extreme ambient conditions, or defence against predators (Kultschar et al., 2018). Although these compounds evolved to bring some life advantages for producing organisms, many of them became toxic for other organisms including humans, and thus represent a threat to all the ecosystem (Sivonen et al., 2010a). Cyanotoxins represent a relatively diverse group of chemical compounds including peptides, alkaloids, polyketides, etc., which can cause damage to prokaryotic cells (antibiotics) as well as to cells of eukaryotic organisms (Kultschar et al., 2018). Several peptides were observed to intoxicate the natural cyanobacterial predators such as *Daphnia* through the inactivation of some of their enzymes (Welker et al., 2006). One of the frequently attacked enzymes in a body of grazer are protein phosphatases, intracellular regulatory enzymes. The inhibition of these enzymes by microcystin can cause serious metabolic disturbances in the grazing organism body. Unfortunately, very similar inhibition effects of these compounds were also reported in the case of vertebrates including mammals (Honkanen et al., 1994). The production of cyanotoxin has been in many cases observed to be associated with formation of cyanobacterial colonies and blooms in eutrophic waters, but there is no obligatory rule for that and both processes can occur separately under different conditions. The exact circumstances, which triggers cyanotoxins production, are still not known (Peterson et al., 2009).

2.1.3. Cyanobacterial metabolism, nutrition

Cyanobacteria belong among autotrophic organisms, which can utilize solar energy to convert carbon dioxide to reduced carbon compounds – carbohydrates. The energy for CO₂ reduction is obtained in the form of ATP macromolecules through photophosphorylation in the photosynthetic apparatus. Cyanobacteria mainly utilize water as a source of electrons for photosystem II, and subsequent electron transport chain. In some conditions, during anoxygenic photosynthesis, other compounds such as hydrogen or sulphide might be oxidized as well (Cohen et al., 1975). The chemical energy gained through the PS is utilized in the Calvin cycle for the conversion of CO₂ to glyceraldehyde-3-phosphate, which is subsequently utilized for gluconeogenesis or energetic metabolism (W. Martin et al., 1997). Besides obligate autotrophs, the cyanobacterial group includes species that can maintain their growth using organic carbon sources. The heterotrophic cyanobacteria can utilize various organic substrates as

a sources of carbon, but the best growth is usually observed when the carbohydrates were employed e.g. cultivation of *Aphanethecemicropica Nägeli* in cassava starch (dos Santos et al., 2017). A mixotrophy, which has been also observed in several species, represents a combination of both heterotrophic intake and phototrophic assimilation of carbon. The mixotrophy can be observed in the case of some facultative autotrophs, which can switch between inorganic and organic carbon sources for biomass production or use an autotrophic regime for biomass formation and heterotrophic regime for intake of some essential compounds (vitamins, cofactors, etc.). Examples of such an organism are marine cyanobacteria *Prochlorococcus* and *Synechococcus* (Duhamel et al., 2018).

Nitrogen is the second most demanded element in cyanobacterial life. Since the body of cyanobacteria contains a higher amount of proteins and nitrogen-based structures (peptidoglycan,..) than higher eukaryotes (López et al., 2010), their growth and reproduction require a constant nitrogen supply. In the case of autotrophic cyanobacteria, the nitrogen is taken in form inorganics forms (ammonium, nitrates, and nitrites) or urea. These compounds can be administrated to the cell either through membrane transporters in ionic forms or partially by passive diffusion in neutral form (depending on the pH of the solution) (Muro-Pastor et al., 2003). As these compounds reach the cytosol, they are converted to ammonia. The nitrates and nitrites special reductases are employed, which reduce these compounds to ammonium using electrons coming from the reduced ferredoxin(Flores et al., 2005)(Sakamoto et al., 2001).A special pathway of nitrogen acquisition is an assimilation of molecular nitrogen from the environment, when the N_2 molecule is reduced to ammonia. This reaction is facilitated by nitrogenase enzyme (Fig.3), which consists of heterotetrametric nitrogenase and homodimer nitrogenase reductase with cofactors a Fe-Mo and Fe respectively (Tsygankov, 2007). The ammonia is subsequently reacting with glutamate upon consumption of ATP and reducing equivalents with enzyme glutamine synthetase resulting in glutamine. From the formed glutamine one amide group is transferred on 2-oxoglutarate via the enzyme glutamine oxoglutarate aminotransferase (Okuhara et al., 1999). As a result of this reaction, two molecules of glutamate are formed, from which one goes back to the first reaction, while the second one is converted by aminotransferases back to the 2-oxoglutarate and any amino acid required(Mehta et al., 1993).The administration of nitrogen to the cell is strictly regulated on both metabolic

and molecular levels. The growth and reproduction of cyanobacteria require a certain C/N ratio, which ensures the growth will proceed correctly. Upon depletion of nitrogen, when the assimilation of CO₂ overtakes the intake of nitrogen, the growth rate is slowed down and the production of storage molecules is triggered (Esteves-Ferreira et al., 2018). In the case of heterotrophs, the nitrogen assimilation goes in the same way as described before for the autotrophs or the nitrogen can be accepted directly in the form of amino acids (Esteves-Ferreira et al., 2018).

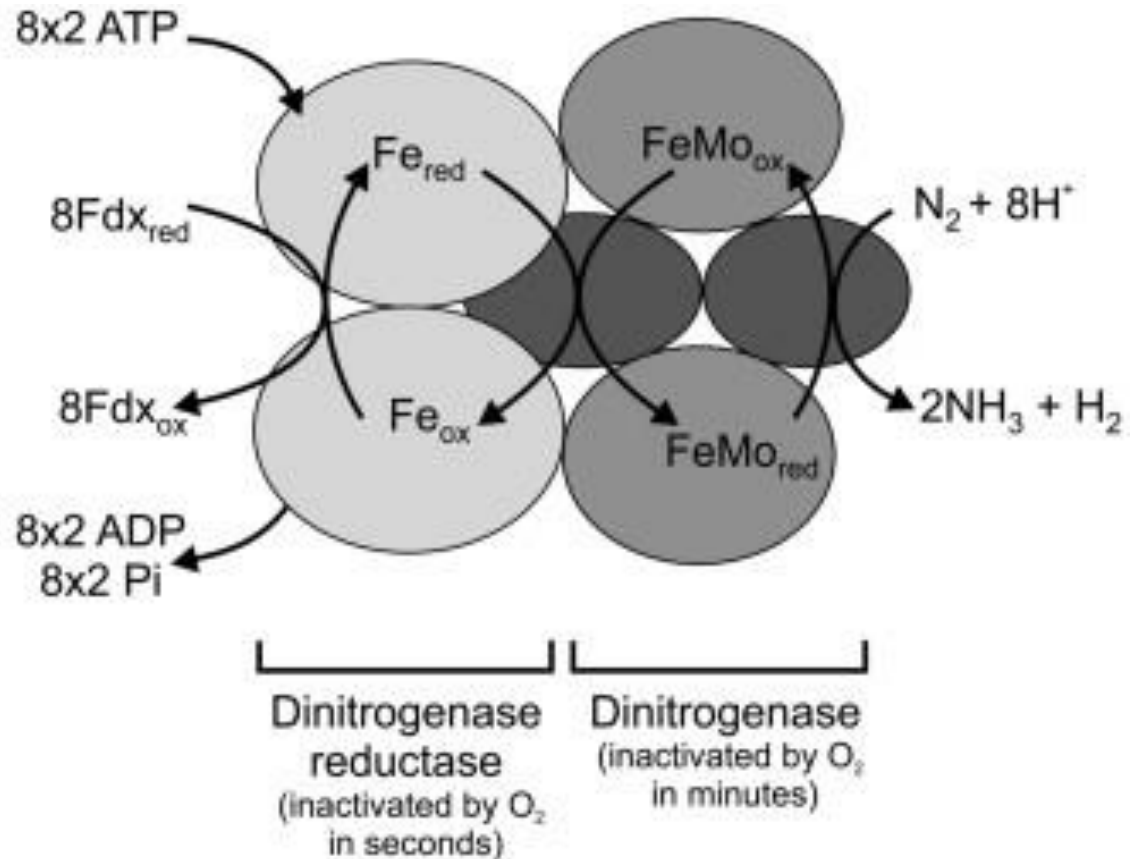


Fig.3.: A working principle of nitrogenase enzymatic machinery found in nitrogen assimilating organisms (Rascio et al., 2018). Abbreviation: ATP- adenosine triphosphate, Fdx- ferredoxin, ADP- adenosine diphosphate, Pi- inorganic phosphate

The carbon and nitrogen are categorized into the group of macronutrients, which work as a building blocks for structural and functional macromolecules and form a majority of dry weight biomass (Markou et al., 2014). Although these macronutrients are essential for the cyanobacterial biomass formation, the provision of solely these elements would not be sufficient for a proper growth and functioning of cyanobacterial cells (Facey et al., 2019). So called micronutrients are also required. The micronutrients consist of alkaline cations (K⁺, Na⁺, Ca²⁺, Mg²⁺) and various metal cations such as Fe²⁺,

Zn^{2+} , Cu^{2+} , etc and represent only a minor part of dry weight biomass (Markou et al., 2014). These cations either play a key role in cell metabolic pathways or are incorporated in many fundamental enzymes and proteins and thus are essential for proper functioning of the cell (Walter et al., 2016)(Shcolnick et al., 2006). Demand for micronutrients in cyanobacteria is elevated in comparison to the other prokaryotes due to the PS apparatus and photophosphorylation, which require many trace metals such as Fe, Mn, Cu, or Mg (Keren et al., 2004)(Keren et al., 2002). Besides the trace metals employed in the PS apparatus cyanobacteria requires also Zn, Co, or in the case of nitrogen-fixing species Mb (Facey et al., 2019).

2.2 Secondary metabolism

2.2.1. Secondary metabolites- characterization

Metabolism can be generally defined as a set of parallel chemical reactions, which occur in the cell to maintain its viability. Based on the products of the metabolic pathway either catabolism or anabolism, is recognized. Besides the biomolecules involved in cellular basal metabolism, anabolism of some organisms is capable of the production of organic compounds, which have no direct function in the primary metabolism. These compounds are generally designated as secondary or specialized metabolites (Bhatla, 2018). Secondary metabolites are a group of chemically diverse compounds, which might be produced by any form of life. As said before, these compounds are not employed in the basal functions of the cell, such as growth, development, and reproduction, but they mediate the abiotic and biotic interactions of the organism with the environment for the purpose to achieve a selective life advantage (Gokulan et al., 2014). The amount of compounds, which are generally considered as secondary metabolites, is tremendous. Up to year 2014 there has been estimated approximately 170 000 natural products originating from either terrestrial or marine organisms (Netzker et al., 2015). In comparison to the primary metabolites, the production of secondary metabolites is not associated with cell growth, but it is either maintained continuously or triggered by the ambient conditions, which influence their lives. A typical example is the production of siderophores (natural iron chelators) as a consequence of a deficiency of free iron in the surroundings (Sandy et al., 2009). An interesting feature is observed in the prokaryotic secondary metabolites where the production is only in poor relation to the taxonomy. A secondary metabolite with a similar or even identical biological activity has been observed to be produced by

organisms from various taxonomical classes with considerable phylogenetical distance. A production of microcystins by phylogenetically unrelated freshwater cyanobacteria such as *Microcystis aeruginosa*, *Hapalosiphon Hibernicus* or *Nodularia Spumigena* might be given as an example (Honkanen et al., 1994). On the other hand, a close relation was observed between the type of produced secondary metabolite and the habitat the organisms are accommodated in. For example, a production of protein synthesis inhibition antibiotics (Erythromycin, Streptomycin, etc.) was observed in gram negative bacteria as well as gram positive bacteria isolated from the same soil samples at the same time (Chandra et al., 2017). Thus, the production of “specialized” metabolites can be associated with a certain habitat the organisms accommodate rather than it would be associated phylogenetically with a certain genus or family.

2.2.2. Secondary metabolites-synthesis and function

The synthesis of secondary metabolites starts with small compounds originating from a primary metabolism such as amino acids, acetyl-CoA, monosaccharides, etc., and is facilitated through many different enzyme complexes and metabolic pathways (Bourgaud et al., 2001). This fact indicates that the synthesis of secondary metabolites is tightly associated with the primary metabolism. An example of such an association might be the synthesis of alkaloids and terpenoids, which directly follows the primary metabolic pathways of aromatic amino acid synthesis or glycolysis/mevalonate pathway (Calabrò, 2015). Alkaloids and terpenoids compounds are secondary metabolites of higher eukaryotes, especially higher plants. Besides these compounds, polyphenols, flavonoids or glycosides are also frequently found in higher plants. The synthesis of these metabolites requires only a few initiating molecules including most notably aromatic amino acids or acetyl-CoA (Bhatla, 2018). The secondary metabolism of fungi represents an “intermediate” state between the secondary metabolism of higher eukaryotes and prokaryotes possessing features of both domains (Keller et al., 2005).

The prokaryotic world is known to be very diverse in the field of secondary metabolites including thousands of compounds of a huge variety. One approach for secondary metabolite production in prokaryotes is classical ribosomal protein synthesis, however with pronounced post-translational modifications (Wilson et al., 2015). Another pathway for peptide based secondary metabolite biosynthesis independent of ribosomes is based on enzymatic apparatus consisting a series of enzymatic modules, which

repetitively utilize the same or similar substrate (Amoutzias et al., 2008). The enzymatic modules themselves consist of 3-5 enzymatic domains, which tightly collaborate on processing of one substrate molecule. A non-ribosomal peptide synthesis (NRPS) and polyketide synthesis (PKS), significant biosynthetic pathways, are working on this principle (Amoutzias et al., 2008). A pathway may consist of one to several dozens modules. The biosynthesis of some cyanobacterial lipopeptides, for example, requires NRPS/PKS enzymatic machinery usually consisting of more than 10 modules (Mareš et al., 2014). The prokaryotic world offers many various ways, how to produce secondary metabolites, but due to their low priority for this thesis, they will not be discussed here.

Secondary metabolites are very diverse group of compounds and the same holds for their ecological functions. They exhibit a very broad scale of functions in the environment and might have a significant influence on ecological interactions, communications among the organisms, or even in biogeochemical cycles (Fig.4)(Sharrar et al., 2020). Within this group we can find many compounds, which play role in competition with other organisms in the surroundings. The competition strategies can involve inhibition of competitors with antibiotics (Grenni et al., 2018), protection of nutrient lines with siderophores (Harrison et al., 2008), degradation of quorum-sensing molecules (Sandoz et al., 2007), and way more. An acquisition of iron using siderophores as natural iron chelators belongs to the processes, where the competition fight frequently takes place (West et al., 2003). This phenomenon will be discussed in more detail in the following chapter.

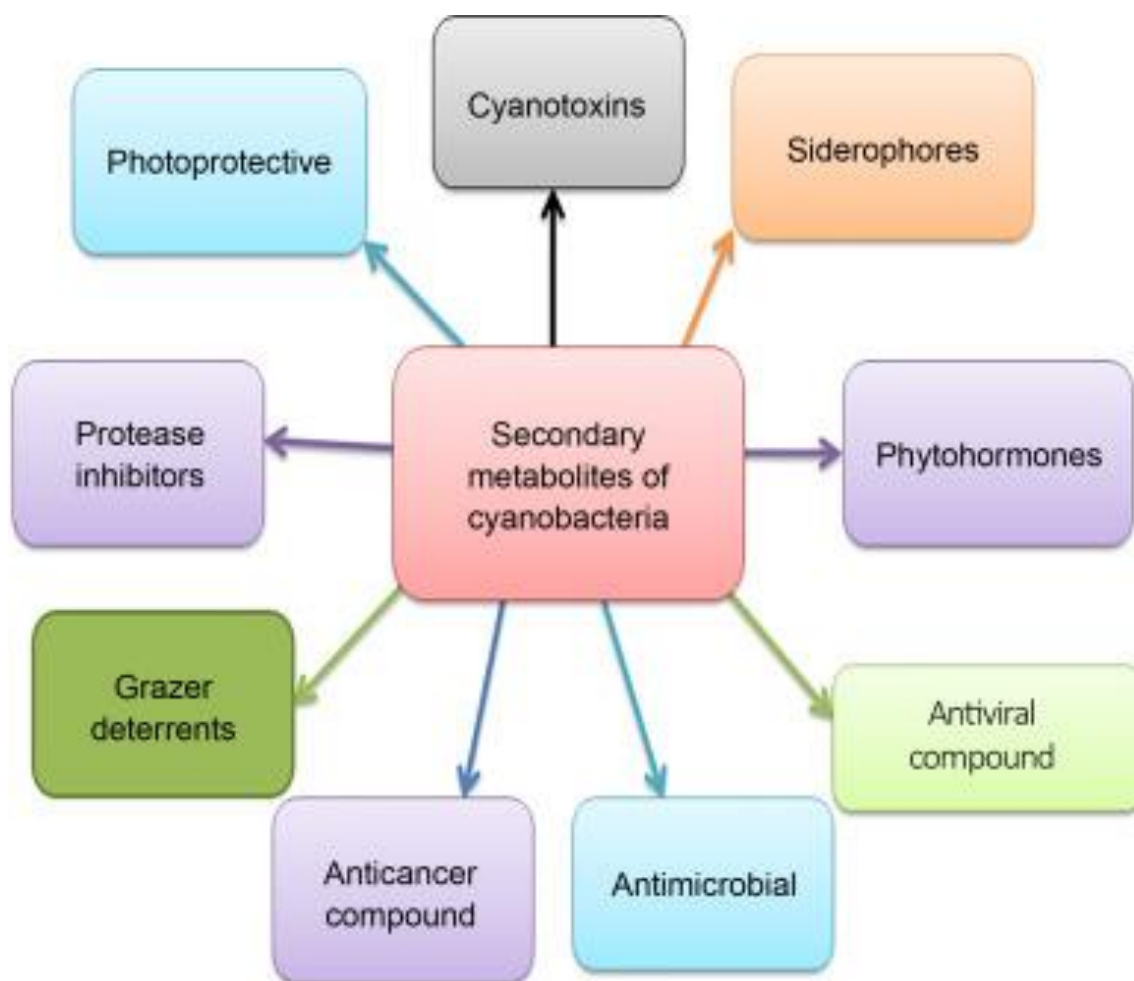


Fig.4.: Various types of bioactive secondary metabolites the cyanobacterial release to the environment

2.2.3.Cyanobacterial secondary metabolites

The cyanobacterial secondary metabolites are usually classified using two different systems. The first system is based on the biosynthetic pathway through which the metabolite is produced, while the second system sorts the secondary metabolite according to their influence towards surrounding organisms. Compounds may fulfil the same ecological role but origins from different synthetic pathways e.g. hepatotoxic compounds microcystin-peptide and saxotoxin-alkaloid (Gademann K et al., 2008).

Ribosomal peptide synthesis represents one of the significant biosynthetic pathways among the cyanobacterial biosynthetic pathways for secondary metabolites production. The production of these compounds starts after transcription of gene clusters of length approximately in range between 5-60 kb, which encode the amino acid sequence of the peptide precursor. In the next step the precursor is tailored with two proteases cutting

the precursor peptide at recognition positions (Schmidt et al., 2005). In following step, a cycle is formed from the α -carbonyl and α -aminogroup at the C-terminal amino acid and the N-terminal amino acid respectively, which yields a cyclic peptide. In a last step of synthesis oxazole or thiazole rings are formed by heterocyclization of some of the amino acids side chains (Sivonen et al., 2010b). At the end of the synthesis some of the amino acids might be modified, which also increases the variability of the products and explains the presence of non-proteogenic amino acids present in the peptide (Gademann K et al., 2008). From an ecological point of view there are many significant ribosomal peptides including already mentioned cyanotoxins such as patellamides and microviridins, which represents a high threat for the organisms in their surrounding (Kehr et al., 2011).

Besides peptides and polyketides, there are also indole alkaloids or isoprenoids, which represent another two important groups of cyanobacterial metabolites. These two groups can also have a significant role in the environment as well as in the cells of their own producers (Huang et al., 2019). The cyanobacterial indole alkaloids are produced by some cyanobacterial species of *Nostocales* order and some of these compounds are categorized as cyanotoxins. A recently discovered Aetokthonostatin produced by, which was found to cause Vacuolar myelinopathy in bald eagles, can be given as an example of neurotoxic indole alkaloid (Breinlinger et al., 2021). The biosynthetic pathway starts with production of indole isonitrile molecule from tryptophan and ribulose-5-phosphate (Walton et al., 2016). The resulting geranyl pyrophosphate molecule is subsequently attached to indole isonitrile and the molecule is further cyclized resulting to several types of alkaloid precursor (Walton et al., 2016). The cyanobacterial isoprenes are on the other hand employed for cell fitness improvement. The benefit of isoprene production is their photoprotective properties. A higher isoprenes-carotenoids are often employed in photosynthetic apparatus as photoprotective and antioxidative agents, which help to reduce the oxidative stress in the cell (Wada et al., 2013). The synthesis of isoprenes (terpenes) uses two precursor molecules-dimethylallyl triphosphate (DMADP) and isoprene diphosphate (IDP). These precursors are assembled to long chains in many possible combinations and subsequently modified by oxidation, cyclization, etc., which explains a huge diversity of cyanobacterial isoprenes (Pattanaik et al., 2015).

2.2.4. Non-ribosomal peptide synthase (NRPS), Polyketide synthase (PKS), synthesis of lipopeptides

The peptides are very frequent bioactive compounds in prokaryotic world. Their bioactivity and potential utilization for society rose an attention of many scientists in 20th century. During the investigation of this very diverse group of metabolites significant differences were observed between the peptides. In some cases, the peptides contained non-proteinogenic amino acids such as D-amino acids or hydroxy acids. Very common were also constituents of amino acids that had undergone extensive modification e.g. acylation, N-methylation, glycosylation, etc. (Kleinkauf et al., 1990). A deeper investigation in origin of these peptides led subsequently to discovery of complex of enzymes called non-ribosomal peptide synthase, which action is based on three main catalytic domains-adenylation domain, thiolation domain and a condensation domain. Besides these main domains, there are another several enzymatic units, which are responsible for modification of incorporated amino acids (Stachelhaus et al., 1995). The adenylation domain (A-domain) initiates the whole synthesis of a new peptide. The function of A-domain is to catalyse adenylation of amino acids, which leads to its activation. The AMP molecule is connected to the carboxyl group on the α -carbon of the amino acid upon decomposition of ATP molecule (Fig.5). This activation step is crucial for a further reaction, which proceeds on the second domain, the thiolation domain (Stachelhaus et al., 1999). The thiolation domain (T-domain) or alternatively called peptidyl-carrier protein (PCP) serves as an intermediate between new incoming amino acid and previously synthesised peptide. The T-domain possesses at its active site a 4-phosphopantetheinyl "arm" residue with thiol function group. The amino acid-adenylate coming from adenylation domain reacts with thiol group upon formation of thioester bond between amino acid and phosphopantetheinyl residue and releasing the AMP (Fig.5) (Weber et al., 2000). Once the amino acid is attached to the phosphopantetheinyl residue, the residue transports the amino acid to the last main domain- condensation domain (C-domain). In the C-domain the amino acid meets another amino acid coming from the downstream module. When the amino acid gets into close contact, the α -amino group on downstream amino acid starts to act as a nucleophile, attacking the thioester bond of the upstream amino acid (Fig.5). As a result the peptide bond between these two amino acids is formed upon releasing of upstream amino acid from the phosphopantetheinyl residue (Keating et al., 2002). A formed dipeptide is transported to the subsequent module, where the peptide is prolonged by a

new amino acid coming from another thiolation domain as described previously. The elongation of the peptide chain goes in this regime further on. At the end of the synthesis the thioester bond is broken by thioesterase and the peptide is released to the cytoplasm (Stachelhaus et al., 1996).

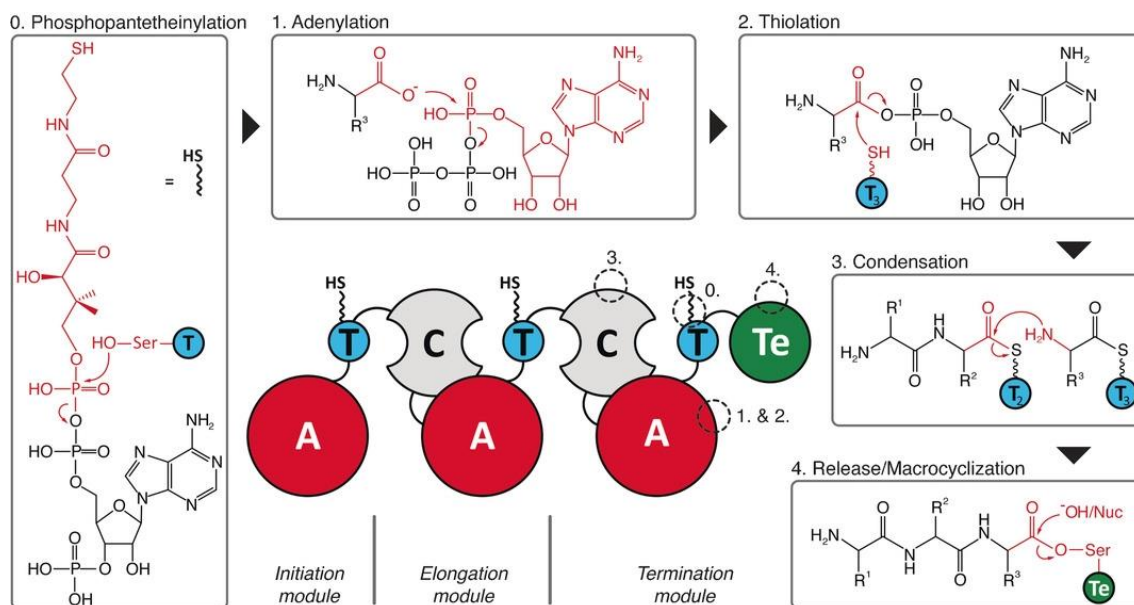


Fig.5.: The chemical reaction included within bacterial NRPS peptide synthesis. 0- NRPS priming: attachment of 4-phosphopantetheine at serine amino acid of the T domain mediated by phosphopantetheine transferase. 1) Adenylation of selected amino acid on adenylation domain using ATP. 2) Formation of thioester bond between activated amino acid upon release of AMP. 3) Condensation of amino acid attached to the T-domain with nascent peptide chain/ amino acid from upstream enzymatic module. 4) Release of nascent peptide from the by thioesterase (Süssmuth et al., 2017). Abbreviation: Ser- serine, Nuc- nucleophile, ATP-adenosine triphosphate, AMP-adenosine monophosphate

These three domains, which were just described, form one “minimal” module or one unit of NRPS synthase. The whole synthase is a large enzymatic complex formed of at least two but usually more modules. The nascent peptide is passing through the complex obtaining one new amino acid in each module (Stachelhaus et al., 1996). Besides the three main domains, the NRPS enzymatic complex contains accessory enzymes, which are responsible for so called “tailoring” of the incorporated amino acids. Among the most important enzymes in tailoring of amino acids belong methyltransferases, aminotransferases, oxygenases, epimerases or heterocyclization domains (Kreutzer et

al., 2012). The tailoring enzymes are one of the reasons, why there are such a high number of structurally different NRPS peptides. Another reason for such a high variability in this class of secondary metabolites is a high amount of adenylation domains, which can incorporate various low molecular substrates with carboxyl group and their promiscuity towards the activated substrates (Leu/Ile/Val, Asn/Gln) (Stachelhaus et al., 1999).

Polyketides can be also found among the prokaryotic secondary metabolites and among the bioactive compounds as well. As an example of bioactive polyketides the macrolides, antibiotic compound, can be given (Kehr et al., 2011). The synthesis of polyketides resembles in many features previously described synthesis of non-ribosomal peptides. Analogous to the NRPS the enzymatic complex in PKS consists of three main catalytic domains, which tightly collaborate on formation of polyketide chain. The first domain in PKS modules is called acyltransferase. This domain catches the malonyl-CoA or acetyl-CoA from the cytosol and brings it directly to the second domain of the module called acyl-carrier protein (ACP) without any preliminary activation (Khayatt et al., 2013). The ACP domain contains as well as in the case of PCP in NRPS a 4-phosphopantetheinyl residue and works in the same principle as in the case of NRPS. The only difference is lack of activation of substrate as it is done in case of NRPS. The reason, why the precursor substrate does not require any activation is the presence of CoA on the utilized substrate, which makes the molecule ready for transfer on the phosphopantetheinyl residue through transesterification reaction (Dunn et al., 2013). The transesterification itself is facilitated by the third domain in the module called ketosynthase. This domain facilitates the formation of the thioester bond between the substrate and phosphopantetheinyl arm as well as the Claisen condensation of malonyl attached to the ACP domain and polyketide chain attached to the ACP one module downstream (Fig.6). The condensation results in prolonged polyketide chain and one molecule of CO₂ (Klaus et al., 2020). As well as in the previously described NRPS the nascent polyketide is passed through several modules prolonging its chain. Except the modules for elongation of the polyketide chain there are very frequently observed auxiliary “tailoring” enzymes, which are incorporated into the modules usually between acyl-transferase domain and ACP domain (Fig.6). Four enzymes are usually involved in the tailoring of the polyketide chain- ketoreductase, dehydratase, enoyl reductase and methyl transferase, but sometimes also aminotransferase or oxygenase can be involved

(Rausch et al., 2007). The involvement of these tailoring enzymes gives a reason for very large amounts of polyketides, which are structurally diverse as it was described in the case of NRPS.

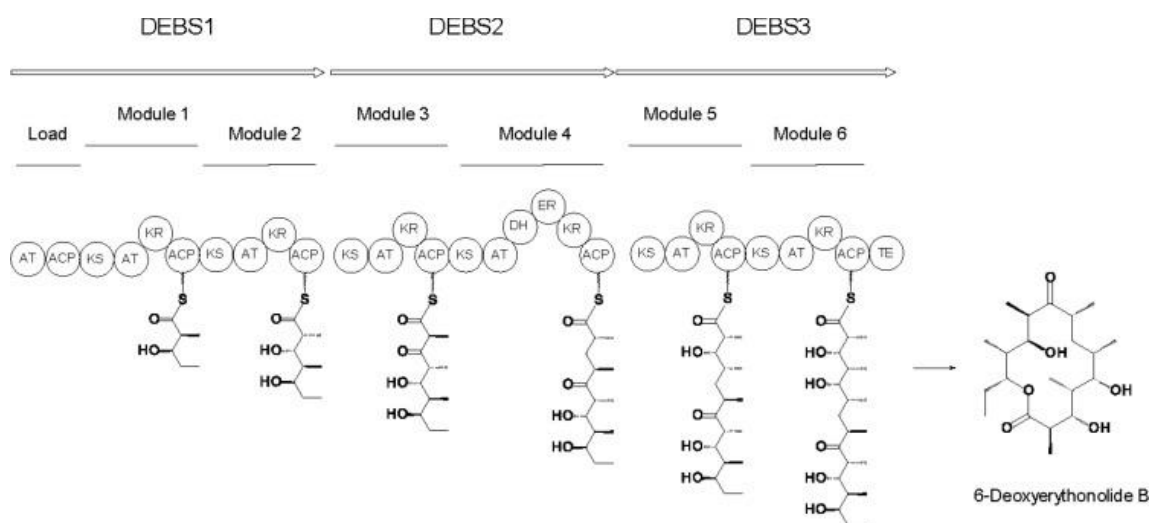


Fig.6.: Demonstration of PKS enzymatic machinery on 6-Deoxyerythronolide B synthase (DEBS). DEBS1- initialization of process by loading module and synthesis of triketide chain including tailoring of the ketide chain by ketoreductase. DEBS2- elongation of nascent ketide chain and tailoring by several types of tailoring enzymes. DEBS3- termination of elongation and tailoring of nascent peptide chain and release from the machine by thioesterase (Caffrey, 2012). Abbreviation: DEBS1-3- 6-Deoxyerythronolide B synthase modules 1-3, AT- acyl transferase, ACP- acyl carrier protein, KS- ketosynthase, KR- ketoreductase, DH- dehydratase, ER- enoyl reductase, TE- thioesterase

In the previous paragraphs the synthesis of non-ribosomal peptides and polyketides was described separately without considering possible connections between these two pathways. But in the world of prokaryotes including cyanobacteria many compounds possessing hybrid peptide-polyketide character can be found. One of these are known as lipopeptides, which are synthesized with collaboration of both biosynthetic pathways (Mareš et al., 2019). The lipopeptide designation was derived from the two main parts of these molecules. The lipopeptides are consisted of peptide chain and hydrophobic moiety. There are several known pathways how the lipopeptides are synthesized, however two pathways are predominant. In both pathways the lipophilic moiety is attached to the single amino acids at the N-terminus of the future peptide chain and the peptide is subsequently synthesized with lipophilic part already attached to the nascent

peptide chain (Duitman et al., 1999). The first known mechanism utilizes the lipidic moiety (mostly some fatty acid) activated by CoA. The CoA is attached to the fatty acid with Fatty acid-CoA ligase. The ligation is accompanied by several modifications, which are performed by tailoring enzymes of PKS origin. The “tailored” fatty is then attached to the amino acid by special condensation domain, which initiates the peptide synthesis (Rausch et al., 2007). The second known mechanism of lipopeptide synthesis works in a similar regime, but the activation is performed through adenylation of the precursor lipid moiety (via Fatty acyl-AMP ligase), again mostly some fatty acid. After the adenylation the fatty acid is again modified by a cascade of tailoring enzymes of PKS origin and brought to the T-domain of the first module of NRPS. The activated fatty acid is then connected to first amino acid at N-terminus of nascent peptide and the non-ribosomal synthesis starts (Mareš et al., 2019) (Duitman et al., 1999). The combination of both previously described biosynthetic machineries with employment of all the tailoring enzymes results again in huge variety of molecules, which are widespread among many significant bacterial strains such as actinobacteria, firmicutes or most of the classes of proteobacteria (Khaledian et al., 2020) and of course cyanobacteria.

The combination of non-ribosomal peptide synthase (NRPS) and polyketide synthase (PKS) pathways gave rise to a biosynthetic pathway for lipopeptide production in cyanobacteria. Lipopeptides are usually amphiphilic compounds consisted of peptide chain of various length and lipophilic moiety. The amphiphilicity of the molecule is usually given mainly by a composition and polarity of amino acids incorporated to the peptide chain and their possible modification by tailoring enzymes (Cochrane et al., 2016). The cyanobacteria can produce lipopeptides in either cyclic or linear forms. There are several groups of cyanobacterial lipopeptides, which are known to form a cyclic structures such as Puwainaphycins (Hrouzek et al., 2012) or Hassallidins (Neuhof et al., 2005). Besides ring forming lipopeptides, the linear lipopeptides such as Microginins (Stewart et al., 2018) or recently discovered cyanochelins can be found among the cyanobacterial lipopeptides.

Cyanochelins is a novel group of cyanobacterial lipopeptides, which is consisted of iron chelating compounds-siderophores. The main features of this group are β -hydroxy aspartic acids, which are incorporated to the amino acid chain. These β -hydroxy aspartic

acids are fundamental components of the siderophores since they form the bond between the iron (III) cation and siderophore (Kreutzer et al., 2012) (Galica et al., 2021).

2.3 Siderophores as natural iron chelators

2.3.1. Iron chemistry in aqueous solution

Iron is a ubiquitous element, which can be found everywhere. It forms approximately 32% of Earth mass and is considered as the most abundant compound on this planet (J. W. Morgan et al., 1980). In the Earth crust the most abundant form of iron are divalent or trivalent cations, which are usually fixed in iron minerals such as iron oxides, hydroxides, sulphides, etc. The iron containing minerals represent a significant source of free iron in the environment. During the weathering of the rocks, the iron is leached from complex minerals (e.g. pyrrhotite or pentlandite) in the form of water soluble salts such as sulphates or carbonates (Kong et al., 2012). The lifetime of the free cations in the water column is usually relatively short. The oxygenic atmosphere cause an oxidation of ferrous cation to the ferric cations through a series of complex reactions affected largely by pH which also affects the final product of ferrous cation oxidation (B. Morgan et al., 2007). The oxidation of ferrous cation is a third order reaction with linear dependence on the concentration of ferrous cation and partial pressure of oxygen and quadratic dependence on concentration of hydroxyl ions. Thus the oxidation become negligible at the lower pH, while it is almost impossible to detect ferrous ions in more alkalic solutions since its oxidation proceeds extremely fast (Millero, 1985). The oxidation of ferrous cations results in formation of ferric ions, which are, however, very rarely observed in aquatic solutions in free form, since most of these cations are rapidly trapped by either inorganic or organic ligands to the complex molecules. In the case of inorganic complexes, the ferric ions usually form oxide or oxyhydroxide complexes, which were formed either already during the ferrous cation oxidation or after ferric cation facilitated water hydrolysis (Hudson et al., 1992). The formed complexes have a high tendency to form water insoluble precipitates. The precipitation of iron complexes causes the most significant losses of iron (III) cations from a water column and thus keeps the concentration of dissolved iron in surface waters at extremely low values around 100 pM (Johnson et al., 1997). Besides the inorganic complexes the iron (III) forms also many organometallic complexes with various organic ligands. There is a broad spectrum of organic ligands, which are involved in iron complexation in aqueous solutions. The formations of water-soluble organic iron

(III) complexes counteract the precipitation of inorganic complexes and reduce scarcity of the iron in water column. The organic iron complexes represents about 99% of total dissolved iron (Rue et al., 1995). The organic iron complexes also play a key role in iron reduction in aqueous solution. A reduction of iron (III) back to the iron (II) may occur, when the organic complex is exposed to the UV light. This reaction cause a partial remediation of more reactive and bioavailable form of iron to the water column and it is an important process in biogeological cycling of iron (Rijkenberg et al., 2006). The complexation has a significant effect on the iron availability in the aquatic environment. As mentioned above the precipitation of inorganic iron (III) complexes reduces the iron accessibility significantly. On the other hand, formation of organic iron (III) complexes promotes the retention of iron in the water column since the organic ligands keeps the iron (III) in dissolved or colloidal forms, which are well accessible to the aquatic organisms. Thus formation of organometallic complexes represents a crucial process for life sustainability in aquatic and especially marine habitats, which have only a limited access to other iron source (Katherine Barbeau, 2006). Besides retention of iron (III) in water column, the organic ligand facilitated photoreduction of iron (III) in aqueous solution stand behind the remediation of free iron (II) in the aquatic ecosystems (Rijkenberg et al., 2006).

2.3.2. Siderophores as organic ligands of iron (III) cations

The siderophores are characterized as the strongest iron (III) chelating molecules, which are secreted by living organisms (Kranzler et al., 2013). The secretion of siderophores was established in late 80's by method utilizing chromazurol sulfonate (CAS), which forms complexes blue colour with iron (III). The colour disappear once siderophore is amended, since the siderophore extracts the iron (III) from the CAS complex due to its strong affinity towards the iron (III) cations (Schwyn et al., 1987). The reason for such a high affinity is the structure of the siderophore molecule. Siderophore molecule is capable to fix the iron (III) molecule through several coordination bond, which are formed within one siderophore molecule. The siderophore structure usually possesses six electron donating atoms, which form the coordination bonds. Thus, often only one siderophore molecule is required to fill the free orbitals of the central Fe atom and form a stable complex (Miethke et al., 2007). In the case, when the siderophore cannot provide all the required electron to form a stable complex, an additional ligand can be involved in complex formation. The nature of the ligand might be inorganic such as

H₂O, but formation of more diverse complexes with increased stoichiometric ratio between ligands and central atom can also occur. As example, formation of iron (III) complexes with rhodoturolic acid follows stoichiometric ratio 2:3, iron: ligand respectively (Carrano et al., 1978).

Another reason of high siderophore affinity towards the iron (III) is an atomic composition of ligations sites on the siderophores. The iron (III) cation represents a strong Lewis acid, which tends to form the coordination bonds with strong Lewis bases such as oxygen atoms are. Thus, the iron (III) has a tendency to be strongly solvated in aqueous solutions (Raymond et al., 2014). On the same principle the iron chelation in siderophores molecules works. As mentioned in chapter 2.2.4. the synthesis of cyanobacterial lipopeptides/ peptides creates through NRPS apparatus peptides chains of various amino acids composition. In following chapter, 2.2.5., presence of polar amino acids is mentioned to be frequently observed in structure of lipopeptides. These polar amino acids have a fundamental role in iron chelation, since they provide the required oxygen atoms with free electron pairs, e.g. chelation of iron (III) by Cupriachelin through β -hydroxy aspartic acids (Kreutzer et al., 2012). The chelation of the iron by oxygen functional groups does not occur through random oxygen functional groups present on the peptide chain but it is facilitated via chemical moieties. These moieties has been observed to be identical in siderophores produced by phylogenetically unrelated species and thus were inaugurated as main parameter for siderophore classification (Sandy et al., 2009). There are four classes of siderophores-catecholates, hydroxamates, phenolates and carboxylates, which were considered to the main classes of siderophores. In these four classes the chelation proceeds solely through one chemical moiety. The further investigations in this field, however, revealed a new class called mix-type, where more chemical moieties are involved in iron (III) chelation within one siderophore molecule (Miethke et al., 2007). The chemical structure of moieties, which can be found in siderophores, is shown on structures of siderophores produced by various prokaryotic species in Fig.7.

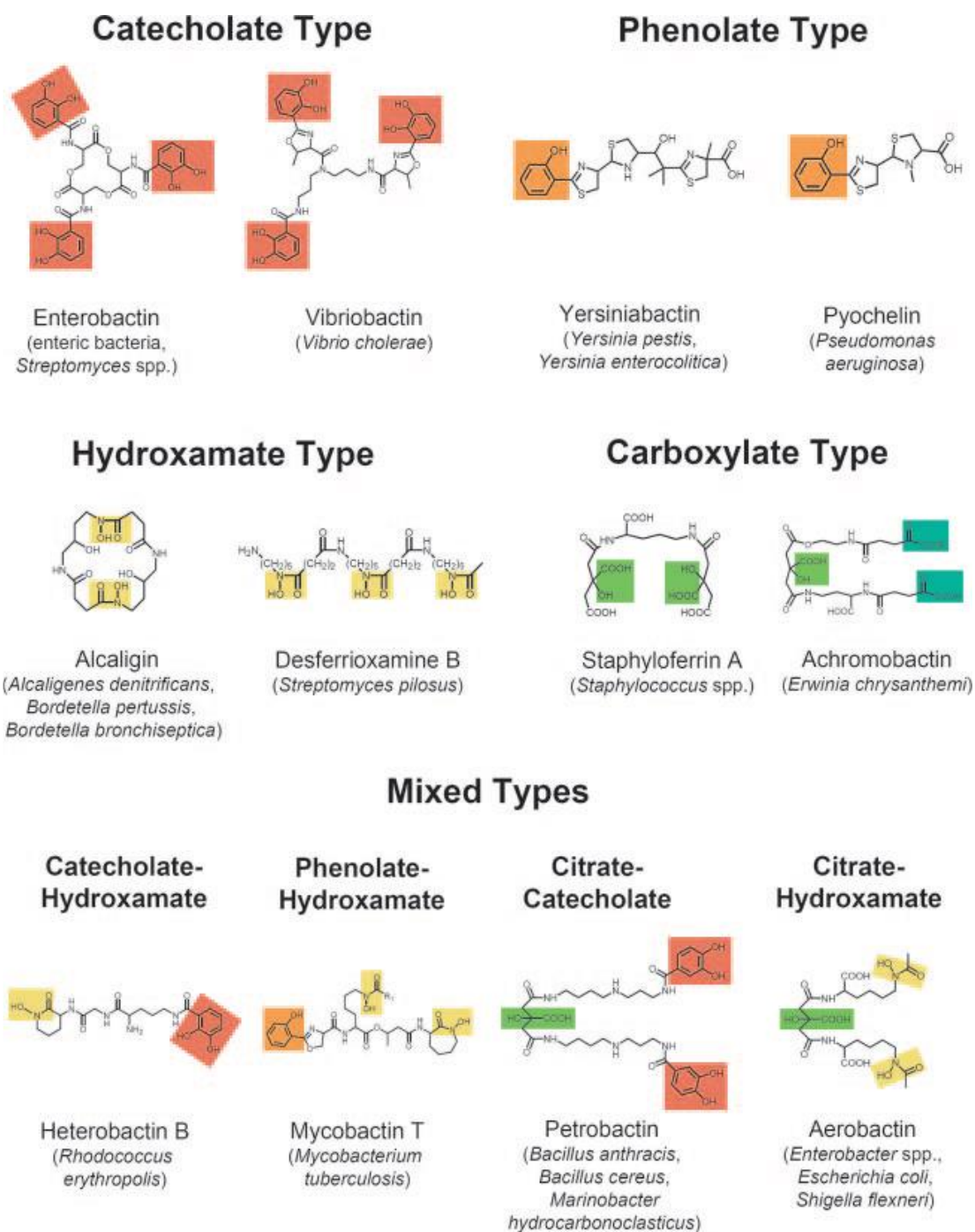


Fig.7.: Representatives of siderophore molecules containing various types of chemical moieties involved in chelation of iron (III) cation (Miethke et al., 2007)

The most frequent moieties observed in cyanobacterial siderophores are hydroxamates, catecholates and carboxylates or phenolates, which are present in the majority of the siderophores, while the phenolates, carboxylates of mixed types can be seen rather rarely (Hermenau et al., 2018). An interesting property of some siderophore-iron (III) complexes is strong absorption in UV spectra. The absorption is not caused by spin-allowed transitions in d-d orbitals of the central atom as it might seem but is caused by a

charge transfer between ligand and metal or ligand and ligand (Miethke et al., 2007). As the absorption is caused by the ligand rather than the central atom, this property is limited to only several types of the siderophores. The siderophores capable of photoreduction will be introduced in chapter.

2.3.3. Cyanobacterial acquisition of iron-non-siderophore mediated uptake

From the biological point of view iron is considered as a one of the most essential micronutrients for prokaryotic as well as eukaryotic cells. This trace metal is a key component in enzymes and proteins involved in cell respiration, electron transport chain, nitrogen metabolism and many other fundamental metabolic pathways. The availability of this element in environment is a limiting factor for carbon and nitrogen fixation and thus represents a crucial element for cyanobacterial life (Lis et al., 2015). Due to the importance of iron cations for cyanobacterial cells several strategies to acquire iron cation have been developed. The uptake of free iron (III) cation can be considered as the easiest way of acquisition. This cation is transported to the periplasm through CDF type TonBs transport channels. In the periplasm the iron (III) cation is subsequently reduced to more biologically active iron (II) by enzymatically produced superoxides and is either transported to the cytoplasm by FeoB transporter (Singh et al., 2008) or back-oxidized to iron (III) and transported to cytoplasm via Fut channel in complex with FutA2 protein. Both pathways were demonstrated on *Synechocystis* PCC 6803 (Kranzler et al., 2011). The mechanism of free iron (III) acquisition can be seen on Fig.8.

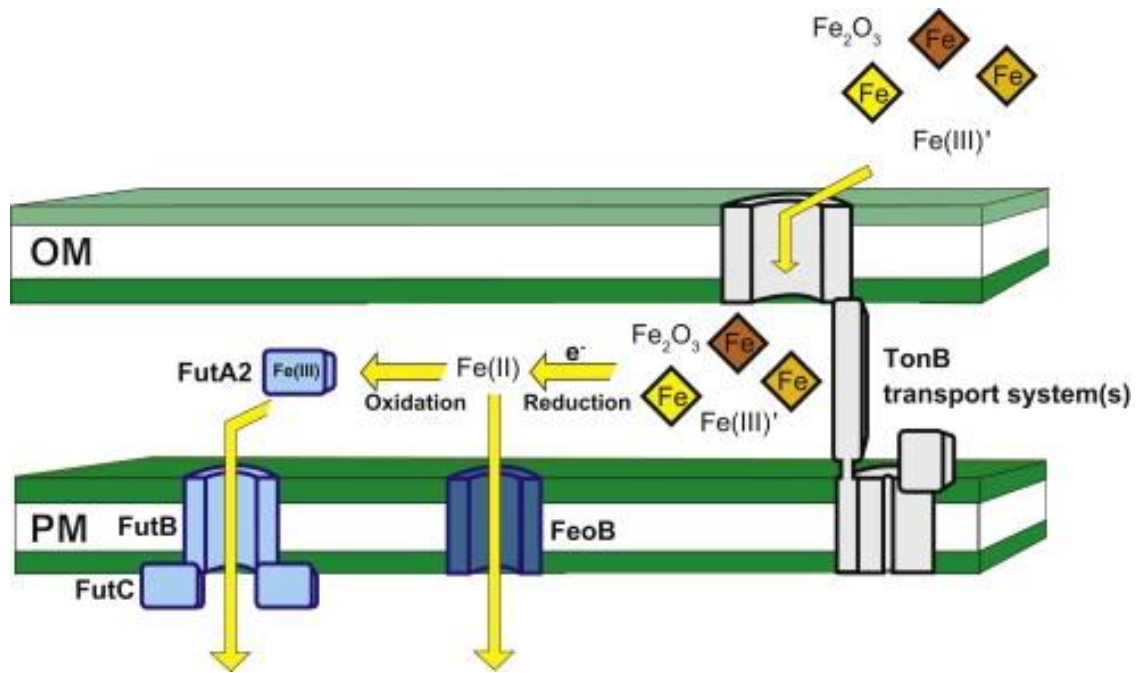


Fig.8.: Mechanism of free iron (III) acquisition observed in *Synechocystis* PCC 6803 (Singh et al., 2008). Abbreviations: OM- outer membrane, PM-plasmatic membrane

The iron (II) can be also taken directly by cyanobacterial transporters, but in an oxygenic environment this sort of uptake covers only a minority of acquired iron (Hudson et al., 1992) (Kranzler et al., 2013). Although a free inorganic iron ($\text{Fe}^{3+}/\text{Fe}^{2+}$) possesses the highest bioavailability for cyanobacteria, the intake of these inorganic forms represents only a minor part of iron intake in cyanobacteria cell since the majority of the environmental iron is captured in complex molecules (Gledhill et al., 1994). One strategy, how the cyanobacteria acquire a complexed iron (III), is called a reductive iron uptake strategy and it is applied on iron (III) organic complexes. Within this strategy the iron (III) is reduced with extracellular superoxides to iron (II). The formed iron (II) losses its affinity to the ligands and is liberated from the complex to the environment and can be directly taken by cyanobacteria (Lis et al., 2009). In the case of iron (II) organic complexes the decomposition of the complexes is not necessary since these complexes can be directly transported to the cell as it was demonstrated on *Lyngbya Majuscula* (Rose et al., 2005). The previously mentioned strategies utilize compounds with reductive potential to increase the concentration of free iron in their cell surrounding followed by subsequent transport of the non-ligated form. Another type of strategies, how to acquire iron from surrounding, is based on acquisition of iron bound by ligands, which are produced by the cyanobacteria or other microbes and are generally called siderophores (Welker et al., 2006).

2.3.4. Cyanobacterial acquisition of iron- siderophore mediated uptake

The iron acquisition is a life sustaining process in cyanobacterial life. Thus, a several strategies were developed to ensure regular supply of this nutrient. Besides strategies based on the intake of free iron from the solution, which were introduced above, cyanobacteria have evolved strategies using strong organic ligands, siderophores (Miethke et al., 2007). These organic ligands can form stable, 1:1 stoichiometric complex with iron (III) molecules, which makes them suitable for iron (III) transport from the source to the cell across the water column. As said at the beginning of this section, siderophores, as the organic iron ligands, prevents the iron precipitation and sedimentation in the water and thus make it more accessible to cyanobacteria (Katherine Barbeau, 2006). Despite their unique properties and significance in nutrition, siderophores are produced only temporally as reaction to iron starvation, which can cause disturbances in many metabolic pathways, photosynthetic and respiration machineries (Sandy et al., 2009). The production of siderophores is an energy consuming process, which requires biosynthetic macromolecules such as amino acids or fatty acids. Thus, the synthesis of siderophores must be regulated by down as well as up regulating mechanisms. The regulation is facilitated on by DNA binding iron (II) dependent Fur proteins (Keyer et al., 1996), anti-sense RNA (Hernández et al., 2006) and by physiological responses of cell to oxidative stress sparked by iron starvation. The regulation is very difficult to describe precisely, since all the listed pathways overlap in both down and up regulation processes (Kranzler et al., 2013).

The produced siderophores are excreted from the cell to the surroundings. Before the siderophores are excreted, they must pass the inner membrane and the outer membrane of the cyanobacteria cells. The transport through the outer membrane is mediated by TolC-like protein, which were observed to be involved in siderophore secretion by *Anabaena* sp. PCC 7120 (Nicolaisen et al., 2010). The transport through the inner membrane is suggested to be mediated by several super families of protein transporters, but the exact protein has not been described yet (Miethke et al., 2007). After they trap iron, the siderophores are transported back by the cell. The uptake goes through specific transporters with high affinity towards siderophores. The TBDTs (TonB-dependent transporters) are 22-stranded β -barrel proteins forming a transmembrane channel embedded in the outer membrane of the cell with N-terminus plug inserted in the pore (Fig.9). The siderophore binds to the pocket of the transporter,

which faces the extracellular space and contains a specific binding site. Subsequently the siderophore molecule is transported to the periplasm (Chimento et al., 2005). The uptake of siderophore is energy consuming process. The energy for the transport to the periplasm is provided by proton motive force from the inner membrane. The protons are transferred via three inner membrane proteins-TonB, ExbB and ExbD, which connects the TBDTs with the cytoplasmic membrane (Higgs et al., 2002). After the siderophore is transported to the periplasm, two situations may occur. The iron is either released from the siderophore complex and is taken as a free element to the cytoplasm as described in previous chapter or is transported to the cytoplasm in complexed form by ABC transporters or permeases. In both situation the transport finishes with reduction of iron (III) to iron (II) by cell produced superoxides and incorporation of the cation to the metabolism (Schalk et al., 2012).

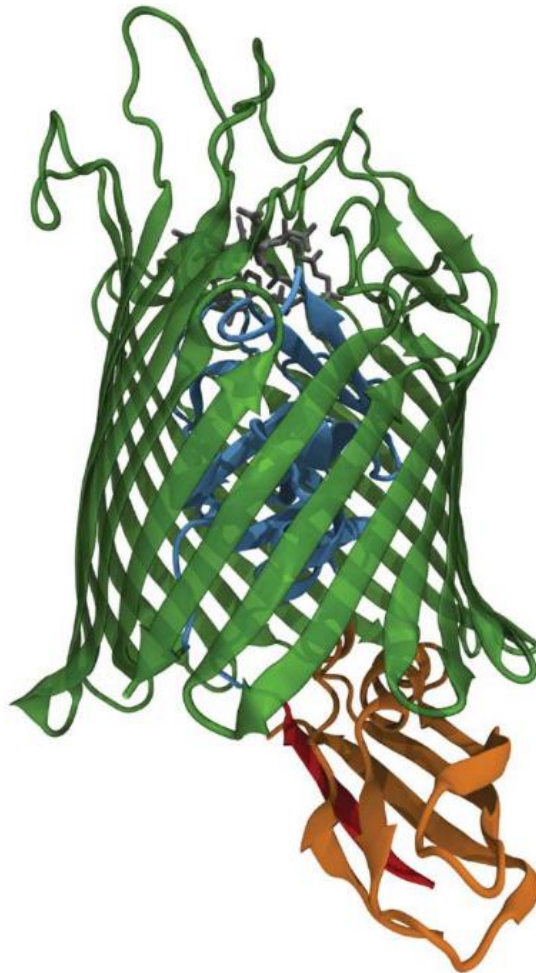


Fig.9.: The structure of TBDT transporter observed in gram-negative prokaryotes. Green- 22-stranded β -barrel embedded in outer membrane, blue- N-terminal plug

anchored to the barrel, orange- TonB3 protein connecting the TBDT with cytoplasmic ExbB transporter (Kranzler et al., 2013)

2.3.5.Siderophore mediated photoreduction of iron (III) cations

The previous chapter described the siderophore mediated uptake of iron (III) to the cell. Although this strategy represents a convenient method for iron (III) acquisition, it is not the only strategy of siderophore mediated iron acquisition. As described above, some of the siderophores are capable to perform iron (III) reduction, when exposed to the light. The mechanism of iron (III) reduction can proceed through the direct way and indirect way (Katherine Barbeau, 2006).

The direct way represents the more common way of siderophore facilitated iron (III) reduction. In this case the reduction is carried on solely within the siderophore-iron (III) complex, when the charge is passed from ligand directly to iron (III) upon exposition of the complex to UV light (Fig.10). The reaction results in formation of iron (II) and it is sometimes accompanied by degradation of the siderophore (Katherine Barbeau, 2006). As mentioned above, the absorption of the complex depends on the type of the siderophore employed. From the three main groups of siderophores types (α -hydroxy carboxylates, catecholates and hydroxamates) the α -hydroxy carboxylates were found to be able to form iron (III) complexes with highphotoreduction potential (Katherine Barbeau et al., 2003). The absorption of these complexes usually reach the maximum at approximately 300 nm as observed in several aquachelins (Martinez et al., 2000), Pacifibactin (Hardy et al., 2019), gramibactin (Hermenau et al., 2018) etc. The photoreduction ability was reported in several reviews to be provided through two different chemical moieties- citric acid and β -hydroxy aspartic acid (Sandy et al., 2009) (Katherine Barbeau, 2006).

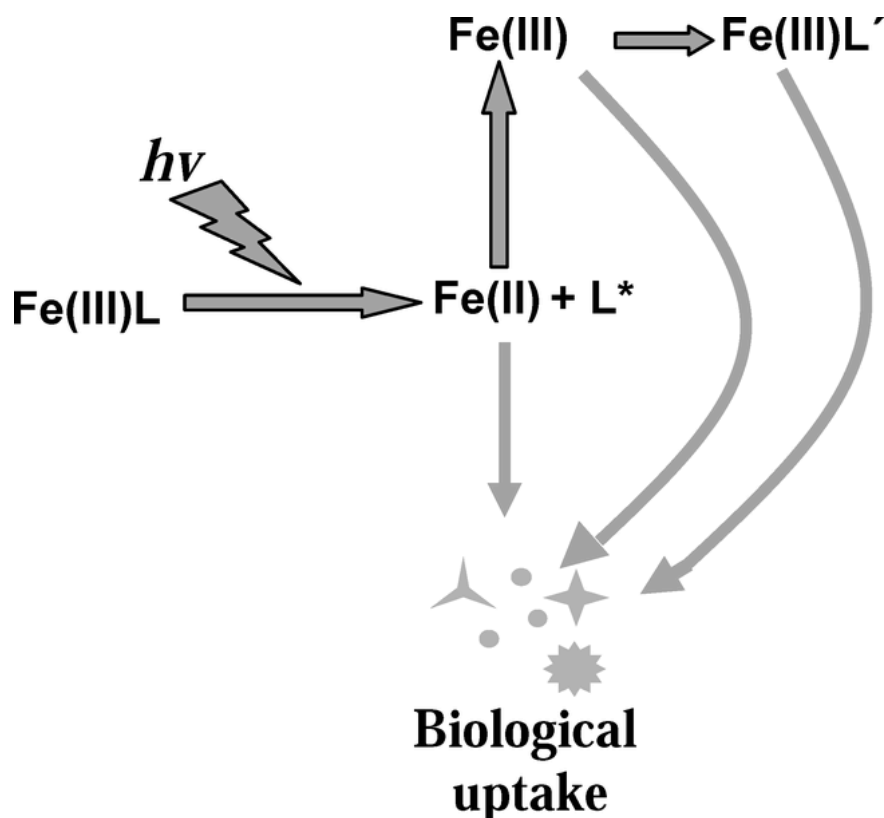


Fig.10.: Mechanism of direct iron (III) photoreduction mediated by photoreactive siderophores and subsequent acquisition of iron in bioavailable forms (Katherine Barbeau, 2006). Abbreviations: $Fe(III)L$ - photoreactive siderophore- $Fe(III)$ complex, L^* - siderophore photoproduct, L' - iron(III) ligand different from original siderophore

The photoreductive activity of citrate containing siderophores has been detected in several siderophores families such as ochrobactins (J. D. Martin et al., 2006), synechobactins (Ito et al., 2005) or aerobactin (Butler et al., 2021). The photoreduction of iron (III) in citrate containing siderophores is accompanied by photooxidation of citrate to 3-ketoglutarate and evolution of CO_2 , which compensates the electron gain of the central atom. The reaction proceeds in equimolar stoichiometric ratio utilizing one ligand molecule per one iron (III) cation. The photoproduct, 3-ketoglutarate, undergoes immediately spontaneous keto-enol tautomerization, which results in water stable enolate form (Butler et al., 2021). The tautomerization of photoproduct results in formation of a new iron (III) coordinating hydroxyl group, which compensates the coordinating group lost during the previous photoreduction. The compensation of coordination group by tautomerization cause that the photoproduct persist the ability to coordinate the iron (III) with similar affinity as observed in the original molecule (Klipper et al., 2006).

Another chemical structure involved in siderophore mediated iron (III) photoreduction is β -hydroxy aspartic acid. There are several families of siderophores, which employ β -hydroxy aspartic acid for iron (III) photoreduction. Besides β -hydroxy aspartic acids these siderophores commonly contain lipidic moiety, which is attached to the C-terminus of the peptide chain (Sandy et al., 2009). Among the known examples of these amphiphilic ligands belong aquachelins, alterobactins, marinobactins (Sandy et al., 2009) or newly discovered cyanochelins (Galica et al., 2021). The photoreduction of the iron (III) is accompanied by the photolytic degradation of the ligand molecule as well as it was described in citrate containing siderophores. In this case the siderophore molecule is affected within the peptide chain at position of β -hydroxy aspartic acid. The reaction mechanism was described precisely in the case of aquachelins (Fig.11); however, a similar mechanism is expected also in the case of other siderophores. The photolysis of iron (III) aquachelins complexes is accompanied by oxidation and cleavage of β -hydroxy aspartic acid from the peptide chain together with fatty acid. The resulting product of aquachelin photolysis still contains two β -hydroxy aspartic acids and so these photoproducts are still capable to coordinate the iron (III) cations, but there is no closer description of this phenomenon. As it was already mentioned, the photoreactivity of β -hydroxy aspartic acid consisting siderophores was demonstrated in many bacterial or cyanobacterial siderophores such as cupriachelin (Kreutzer et al., 2012), pacifibactin (Hardy et al., 2019), aquachelins (Martinez et al., 2000) and cyanochelin 1026 (Galica et al., 2021)., however, there is only a little known about how the kinetics of the photoreduction and activity of the photoproducts (Butler et al., 2021).

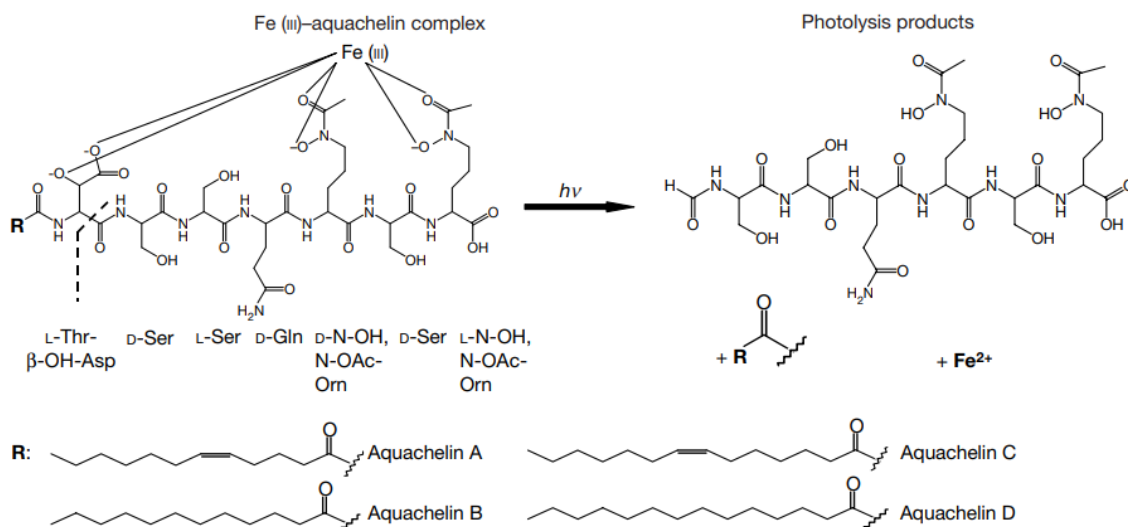


Fig.11.: Representation of photochemical degradation of aquachelin-Fe(III) complex upon photolytic cleavage of aquachelin molecule and release of iron(II) cation. Dashed line- the position of aquachelin molecule cleavage (K. Barbeau et al., 2001)

2.3.6. Cyanochelin m/z 1026

The recent work on the field of cyanobacterial iron acquisition strategies has revealed a new class of cyanobacterial siderophores called cyanochelins. As already mentioned in chapter 2.2.5, these NRPS/PKS synthesised lipopeptides are α -hydroxy carboxylate-type siderophores with β -hydroxy aspartic acids incorporated to their peptide chain (Galica et al., 2021). The cyanochelin m/z 1026 is a siderophore, which was identified to be produced by soil cyanobacterium *Leptolyngbya* sp. strain NIES-3755 upon iron starvation. The structure of peptide chain was estimated by high-resolution mass spectrometry (HRMS) to be consisted from 6 amino acid- [Phe]-[Gly]-[β -OH-Asp]-[Ser]-[β -OH-Asp]-ethanolamine (Galica et al., 2021). Due to the β -hydroxy aspartic acids present in the peptide chain the cyanochelin m/z can coordinate the iron (III) cations. The four out of six coordination bonds are provided by the β -hydroxy aspartic acids, the chelation atom of the third coordination bond is still not known exactly. The iron (III) complexes formed through β -hydroxy aspartic acids have a strong absorption band around 300 nm. In the case of pacifibactin a significant characteristic absorption was measured at 305 nm (Hardy et al., 2019). In the case of cyanochelin m/z 1026 two absorption peaks were identified at 254 nm and 310 nm. The absorption bands in near UV-light indicates the photoreductive potential of these siderophores, since the UV light provides the energy required for electron transfer between the siderophore and centra

iron (III) atom (Miethke et al., 2007). Despite the photoreactivity of cyanochelin m/z 1026 iron (III) complex was confirmed by preliminary test with photometric method using bathophenanthroline disulfonic acid, there is no knowledge about the kinetic of the reaction. The HPLC-MS/MS analysis of reaction solution after exposition to the UV light revealed formation of photoproduct m/z 633 of cyanochelin m/z 1026 photolysis (Galica et al., 2021), but so far no further investigations were carried out.

3. The aim of the work

- Optimisation of procedure for isolation of cyanochelin 1026
- Isolation of sufficient amount of cyanochelin 1026 required for following experiments and structural analysis of the molecule (NMR)
- Evaluation of kinetics of cyanochelin 1026-Fe³⁺ complex photodegradation
- Identification of photolytic fragments of cyanochelin 1026
- Determination of reaction stoichiometry of cyanochelin 1026-Fe³⁺ complex photodegradation in excess of iron (III)

4. Materials and methods

4.1 Cultivation of cyanobacteria *Leptolyngbya* sp. NIES-3755

Cyanobacterium *Leptolyngbya* sp. NIES-3755 was grown at room temperature, supplied with constant gas input with 1,5% vol. CO₂ concentration (Mareš et al., 2019), and exposed to the constant illumination of 4 μmol photons m⁻².s⁻¹ in form continuous dispersed light generated by LED strips (warm white). The manipulation with the biomass was carried out in horizontal laminar flowbox using flame sterilization. The cultivation was carried out in 5 L cultivation flasks, which are after sterilization sealed with flame sterilized cellulose plug equipped with glass tube crossing the plug in the middle for gas input. Approximately 3,5 L of *Leptolyngbya* culture was grown in one container. Inoculum was provided from one of NIES 3755 cultures routinely maintained in the lab of the supervisor. For siderophore production, the cultivated biomass was transferred to the iron deficient BG-11 media (BG-11-Fe). The biomass was exposed to the iron-deficient media for approximately four weeks, which triggered the siderophores production and subsequent release to the media (Sandy et al., 2009). The presence of siderophores was tested with CAS assay (chapter 4.2.1.). In the case the presence of siderophore was proved, the biomass and the medium were harvested.

To obtain sufficient amount of induced biomass for siderophore extraction the biomass was cultivated in large, semisterile 15L flat-panel photobioreactor at room temperature and light of irradiance 238,7 μmol m⁻².s⁻¹. In the case of semi-sterile mass cultivation, 5L of dense culture grown in sterile environment was transferred to the 15L flat-panel photobioreactor (see Fig.12), which was pre-sterilized with chlorine-based bleach (NaOCl). The culture was subsequently diluted up to 12L with iron deficient media (BG-11-Fe). After two weeks the media was cautiously removed by suction from the surface of the flat-panel photobioreactor after the sedimentation of *Leptolyngbya* sp biomass, which was caused by temporary interruption of gas flux coming out of the tube on the bottom of the flat-panel photobioreactor. The removed media was replaced by fresh BG-11-Fe and the culture was exposed to iron deficient environment for another week. At the end of the third week the culture was tested for siderophore occurrence and harvested.

Tab.1.: The composition of BG-11 medium used for cultivation of *Leptolyngbya* sp. strain NIES-3755

Substance	Volume [ml]
BG-FPC solution	10
6g.l ⁻¹ Ferric ammonium citrate	1
0,188 M Na ₂ CO ₃	1
0,175 M K ₂ HPO ₄	1
H ₂ O	987
BG-FPC composition	
Substance	Amount
NaNO ₃	37,4 g
MgSO ₄ x 7 H ₂ O	1,87 g
CaCl ₂ x 2 H ₂ O	0,9 g
C ₆ H ₈ O ₇ citric acid	0,15 g
0,5 M EDTA	140 µl
Microelement solution	5 ml
H ₂ O	250 ml
Microelement solution composition	
Substance	Amount
H ₃ BO ₃	2,86 g
MnCl ₂ x 4 H ₂ O	1,81 g
ZnSO ₄ x 7 H ₂ O	0,22 g
Na ₂ MoSO ₄ x 2 H ₂ O	0,39 g
CuSO ₄ x 5 H ₂ O	0,079 g
Co(NO ₃) ₂ x 5 H ₂ O	0,049 g
H ₂ O	250 ml

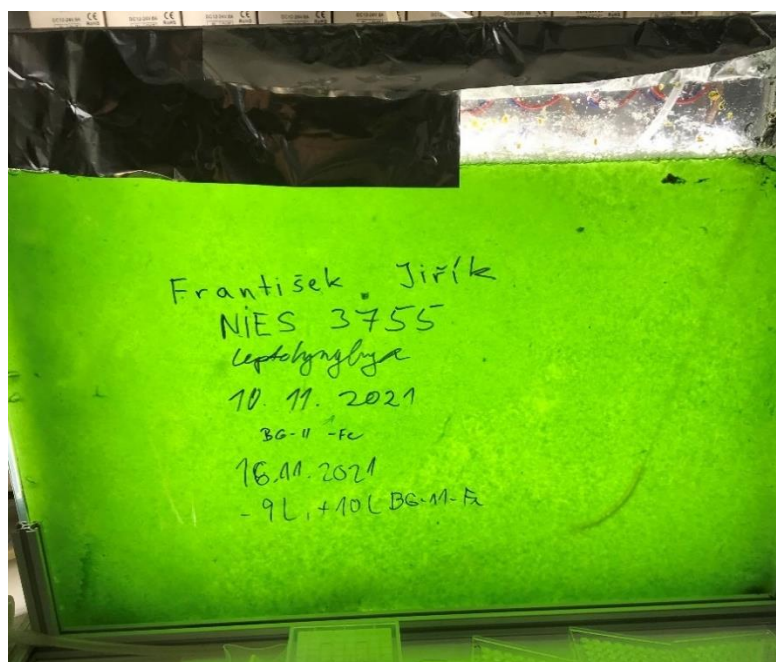


Fig.12: Semi-sterilized *Leptolyngbya* cultivation in flat-panel photobioreactor

4.2 Cyanochelin m/z 1026 harvesting

4.2.1. CAS assay

The CAS assay is a spectroscopic analytical method based on Fe^{3+} cation exchange between complex of Chrom Azurol S (CAS Nbr. 70-51-9) with Fe^{3+} cation and hexadecyltrimethylammonium bromide-HDMTA (CAS Nbr. 57-09-0) (further only CAS complex), and siderophore. Due to the high affinity of siderophore to Fe^{3+} cation the Chrom Azurol S – Fe^{3+} complex is disassembled, which is accompanied by colour change of the solution from blue to yellow, which signalize the siderophore presence (Schwyn et al., 1987). The assay was used to monitor the siderophore production in *Leptolyngbya* sp. cultures.

For CAS preparation procedure 1mM solution of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ was prepared in 10mM HCl. In the first step 0,75 ml of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution was mixed with 3,75 ml of 2mM Chroma Azurol S creating a mix, which was subsequently added slowly to 3 ml of 10mM HDMTA solution in 50 ml Falcon flask upon continual stirring. For the following step the aqueous solution of piperazine was prepared, using 2,150 g of piperazine dissolved in 8,2 ml 4,6M HCl. In the last step the piperazine solution was mixed with the Chrom Azurol S solution in the Falcon tube and the tube is filled up to 50ml with distilled H_2O . The CAS assays were performed on 96-well plates, where 100 μl of solution containing CAS complex was mixed with equal volume of the harvested medium. The presence of Siderophore is confirmed by change of the solution colour, which usually occurs within several minutes after the application.

4.2.2. Optimisation of cyanochelin m/z 1026 extraction from dried biomass

To perform the efficient extraction of cyanochelin m/z 1026 from the biomass initially methanol and acetonitrile solutions of different percentages (0, 20, 40, 60, 80 and 100%) in water were tested. The extraction from dried biomass was performed in the following procedure. The biomass sample was mixed with solvent mixture in ratio 1 ml solvent to 20 mg of dried biomass and extracted by sonication in an ultrasonic bath for 15 minutes. Homogenized mixture was subsequently centrifuged (5 minutes, 14 100xg) and the supernatant is removed. This procedure was repeated in three times and after each centrifugation, the sample from supernatant is taken for the quantitative HPLC- MS/MS analysis as described in chapter 4.3.

4.2.3.Extraction of cyanochelin m/z 1026 via liquid-liquid extraction using solvent of different pH

The cyanobacterial biomass was extracted with 60% ACN in the ratio 1ml 60% ACN to 20 mg of dried biomass based on the results from extraction optimisation experiment. The mixture was subsequently treated in ultra-sonification bath for 15 minutes and centrifuged in 50 ml centrifugation tubes for 15 minutes at 4000xg. The supernatant was then transferred to a separate beaker. The pellet was treated with fresh 60% ACN and extraction was repeated in the same manner as in the first step. All together the extraction was repeated three times. After the third extraction step the pellet was discarded. The collected supernatant containing the compound of interest was subsequently treated using liquid-liquid extraction. The volume of crude extract was determined in volumetric cylinder and the supernatant was diluted with additional 60% ACN in volume corresponding to approximately 1/3 of the volume of supernatant. After the dilution the pH in the supernatant was increased to 10 with 1M NaOH. The crude extract was then mixed with equal volume of ethyl acetate (EtAc) and the mixture was shaken thoroughly in a separation funnel. After the phase separation the water and organic phases were collected in separated beakers. A 1 ml sample for MS analysis was taken from both. The upper organic phase was discarded. The volume of water phase was determined in volumetric column. The lower water phase containing the siderophore was then acidified with formic acid (88% in water) up to pH=3 and transferred to the separation funnel. An ACN/EtAc (2:3 vol. respectively) mixture was used, which was mixed with water phase in final volume ratio 3:1. The extraction mixture was again shaken thoroughly and left to achieve the phase separation. The phases were then collected separately and sampled for HPLC-MS/MS analysis (chapter 4.3). Due to the acidic pH the siderophore is now in the organic phase. This extraction step was subsequently repeated using the water phase collected from the previous extraction. The collected organic phases are subsequently concentrated on rotary vacuum evaporator under pressure reduced to approximately 900 mbars. The concentrated samples are prepared for preparative HPLC.

4.2.4.Isolation of cyanochelin m/z 1026 with preparative HPLC

The compound of interest was isolated via reverse phase HPLC separation performed on Agilent 1100 Serie chromatograph equipped with DAD (Diode-Array-Detection) module. As a stationary phase a μ Bondapak Phenyl Prep (10 μ m, 125Å, 7,8x300 mm)

column was used. The composition of a mobile phase was 25% acetonitrile as solvent A and 100% acetonitrile as solvent B. Both mobile phases were acidified with trifluoroacetic acid (Sigma Aldrich, MS grade) to pH $\approx$ 2,4. The gradient used for the isolation is described in Tab.2 and was operated at a constant flow rate of 4 ml/min. Under these conditions the retention time of cyanochelin 1026 is 18-20 minutes. The detection of siderophore was based on its absorption in UV-Vis spectra, particularly at 254 nm with reference wavelength at 290 nm.

Tab.2.: The concentration gradient for pre-HPLC isolation of cyanochelin m/z 1026 molecules

T (min)	B (%)
0	0
1	0
6	30
28	68,6
30	100
35	100
36	0

The isolated fraction containing cyanochelin m/z 1026 was tested for purity using HPLC-MS/MS detection (chapter 4.3). Based on the purity check the isolated fraction were concentrated using rotary evaporator and purified using again prep-HPLC. As a mobile phase, 25% methanol was set as a component A and 100% methanol was set as a component B. The flow rate had fluctuation change during the run between 3,5-4,0 ml.min⁻¹. The gradient used for purification is described in Tab 3.

Tab.3.: The concentration gradient for prep-HPLC purification of cyanochelin m/z 1026 molecules. Abbreviations: T-time, B-mobile phase B, Q-volumetric flow rate

T [min]	B [%]	Q [ml.min⁻¹]
0	0	3,5
1	0	3,5
8	62,5	4,0
30	72,5	4,0
39	100	4,0
45	100	4,0
46	0	3,5

The purified fraction containing cyanochelin m/z 1026 is subsequently concentrated on rotary evaporator and then freeze-dried. The dried siderophore was then dissolved in MeOH, transferred to the pre-weighted MS vial and dried under nitrogen gas flow. At the end of the isolation the vial with siderophore was weighted on Sartorius R 160 P Electronic Semi-Microbalance and the yield of the siderophore was determined.

4.3 Analytical methods

4.3.1. HPLC-DAD analysis

To assess the kinetics of 1026-Fe photolysis reliable quantification method was required. Based on the previous experience with preparative HPLC, which was used during the cyanochelin 1026 isolation, we have decided to monitor the concentration of cyanochelin 1026 by HPLC-DAD. The HPLC analyses were performed on Agilent 1260 Infinity II liquid chromatograph. The mobile phases were used the same as for isolation of the cyanochelin 1026 (phase A- 25% ACN with TFA, phase B-100% ACN with TFA) at a flow rate of 0,6 ml.min⁻¹. The phase gradient is closely described in Tab.4. The injection volume of the samples and calibration standards was 40 µl. As stationary phase, we used an analytical Phenomenex-KinetexC-18 column (2,6 µm, 100 Å, 150 × 4,6 mm), but after several preliminary analysis its use was found inappropriate due to the long retention of the compound on the column, which caused significant losses of the analytes during the analysis and associated unpredictable carryover. After this experience the column was replaced by analytical Phenomenex-Kinetex pentafluorophenyl (PFP) column (2,6 µm, 100 Å, 150 × 4,6 mm), which subsequently shown much better retention of the analytes and generally very reliable peak resolution. The change of the column has a consequence on the retention time, which has changed to approximately 15 minutes. All the samples collected within the UV exposition experiment were analysed in one sequence. At the beginning of the sequence analysis and after every fourth sample a blank (MeOH) was incorporated into the sequence to ensure a regular cleaning of the column. The amount of cyanochelin 1026 in samples was measured as peak area of 254 nm wavelength with reference wavelength 290 nm at retention time 15,4 min. The concentration of the cyanochelin 1026 was subsequently derived from calibration curve prepared in range 150-6,25 µM from solutions of cyanochelin 1026 in MeOH.

Tab.4.: Chromatographic gradient for analysis of cyanochelin 1026 in samples from photolytic reaction. Abbreviation: T-time, B-mobile phase B, Q-volumetric flow rate

T [min]	B [%]	Q [ml.min ⁻¹]
0	0	0,6
1	0	0,6
20	100	0,6
25	100	0,6
30	0	0,6
33	0	0,6

4.3.2.Optimisation of cyanochelin 1026 detection in HPLC-DAD analysis

During the analysis of samples from first experiment the samples were losing significant amounts of cyanochelin 1026 in time. The most probably the problem is a low solubility of amphiphilic cyanochelin in the aqueous solution, which causes sedimentation of the cyanochelin of the bottom on walls of the vials. This problem was fixed by the change of the pH of samples and subsequent dilution of samples with MeOH in 1:1 volumetric ratio to increase the solubility of 1026-Fe complexes and treatment of the samples in ultra-sonic bath for 15 minutes before the analysis. The pH of the samples was changed to pH≈10 using 1M NaOH, which was added after the sample was collected from the reaction solution. This treatment approach was tested in pilot experiment to elucidate its reliability.

4.3.3.HPLC-MS/MS analysis

The high-pressure liquid chromatography with tandem mass spectrometric detection (HPLC-MS/MS) was used for compound identification and quantification. This method was applied for studding the quality of isolated fractions of cyanochelin 1026 as well as during the determination of kinetics of cyanochelin-Fe complex decomposition and identification of photolytic fragments. The compound separation was performed using reverse phase HPLC system Dionex Ultimate 3000 with either phenyl column (μBondapak Phenyl, 10μm, 125 Å, 300x7.8mm) utilized for quality control of isolated fractions or C-18 column (Kinetex, 2,6 μm, 100 Å, 75 × 4.6 mm) for kinetics experiments. The composition of a mobile phase was dH₂O as solvent A and 100% ACN as solvent B. Both mobile phases were acidified with trifluoroacetic acid (0.002% of TFA in ACN and 0.1% of TFA in water). The gradient of mobile phase is described in Tab. 5.

Tab.5.: The concentration gradient for HPLC-MS analyses of cyanochelin m/z 1026 molecules. Abbreviations: T-time, B-mobile phase B, Q-volumetric flow rate

T [min]	B [%]	Q [ml.min⁻¹]
0	15	0,6
1	15	0,6
25	100	0,6
30	100	0,6
33	15	0,6

The HPLC system was connected to qTOF high resolution mass spectrometer (HRMS) with electrospray ionization (ESI-qTOF) Bruker Impact HD II. The settings used for MS/MS analysis are shown in Tab.6. The instrument was calibrated with CH₃COONa clusters before the start of the analysis. The spectra were collected in the range between 100 and 1,500 m/z with a 0.5 Hz spectral rate.

Tab.6.: Parameters of high-resolution mass spectrometer (ESI-qTOF) applied for identification of cyanochelin 1026 molecule

Parameter	Value
dry temperature	200°C
drying gas flow	12 L/min
nebulizer	3 bar
capillary voltage	4,500 V
endplate offset	500 V
Collision-induced dissociation (CID)	20-80 eV

The evaluation of data was performed in the Bruker Compass Data Analysis software by Bruker Daltonic, GmbH. The obtained data were inspected for the presence of peak responding to 1026 m/z ratio (mass of Siderophore molecule is M=1025 Da) via the extracted ion chromatogram function. Using the manual integration program, the area of the peak was calculated, which was directly proportional to the amount of cyanochelin m/z 1026 present in a particular sample.

4.4 The decomposition of cyanochelin m/z 1026-Fe³⁺ complex (1026-Fe complex) upon exposition to the UV light

According to the previous work focused on cyanochelin m/z 1026 (cyanochelin 1026), this siderophore is capable of Fe³⁺ reduction, however, the reaction process has not been described yet (Galica et al., 2021). In this experiment the concentrations of reactants were set default to be equimolar, because only 1026-Fe complexes are demanded to be in the reaction mix with no extra free Fe³⁺ ions. The photolysis was performed in one stock solution in 5 ml Eppendorf tube. Both reactants were prepared in 100 μM concentrations from appropriate stock solutions. The pH of the reaction solution was adjusted to neutral value (pH≈7) using a 2mM 3-(N-Morpholino)propane sulfonic acid solution that was selected according to the literature, where it was employed during a similar experiments (Hardy et al., 2019). The preparation of reagents stock solutions is described in Tab 7.

Tab.7.: Preparation of reagents stock solutions from respective compound

Compound	Physical state	Dilution	Stock solution concentration
Cyanochelin 1026	10 mM MeOH solution	No dilution required	10 mM
Fe(NO ₃) ₃ analytical standard (Sigma Aldrich)	1000 g/l solution in 0,5M HNO ₃	580 μl Fe(NO ₃) ₃ 420 μl dH ₂ O	10 mM
3-(N-Morpholino)propane sulfonic acid(MOPS) (Sigma Aldrich)	Solid white powder	10,47g MOPS 50 ml dH ₂ O 10M NaOH-pH adjustment	1 M

The reaction solution was prepared in 3 ml volume by addition of 2940 μl of MOPS solution (2 mM). After the MOPS was administered to the Eppendorf tube the 30 μl of Fe³⁺ stock solution (10 mM) was amended, and a reaction solution was mixed. Before the addition of cyanochelin 1026 to the reaction solution Eppendorf tube was covered with aluminium foil to prevent precocious exposition to the surrounding light. After covering the Eppendorf tube 30 μl of cyanochelin 1026 stock solution was added to the

reaction solution. After addition of cyanochelin 1026 the reaction solution was mixed and left standing over night at room temperature ensure formation of 1026-Fe complexes (Galica et al., 2021). After the complexation is achieved, the reaction solution is exposed to the UV light.

To perform the photolysis correctly the compatibility between the emission spectra of available UV lamps and absorption spectra of 1026-Fe complex must be ensured. The absorption spectra of 1026-Fe complex in wavelength range 190-750 nm was measured on spectrophotometer Shimadzu UV-2600. The absorption spectrum of MOPS buffer was also measured as well to identify any possible interferences in advance. In both case a distilled water was used as a reference. The measured absorption spectra are presented in the result section. The emission spectra of available LED diode panel containing alternating stipes of Vis and UV diodes were measured with the PSI SpectraPen mini, which measures in the wavelength range 300-900 nm. The light spectra were measured in three different intensities, which reflects the maximum, intermediate and minimal power of the LED panels (Fig. 13).

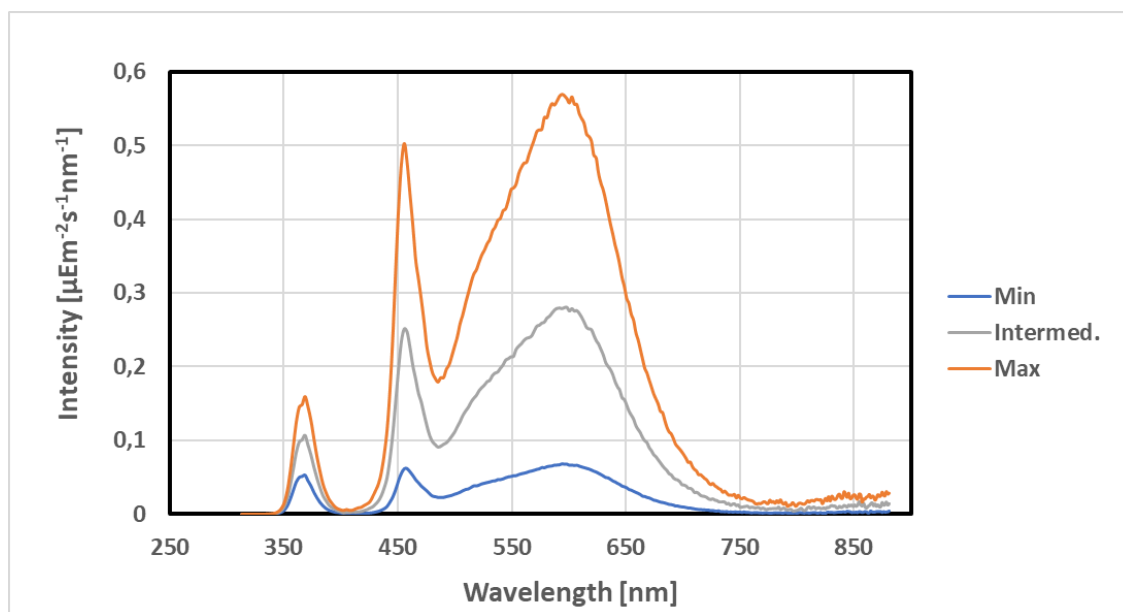


Fig.13.: Emission spectrum of UV-Vis diode panel used for the complex degradation experiment

Based on this measurement the emission spectra of available VU-Vis LED diode panel were considered compatible with absorption spectra of 1026-Fe complex and the following photolytic reactions were feasible.

Within the photolytic experiment the reaction solution, which was prepared according to the procedure described above, was exposed to the full spectra light coming from the UV/Vis LED diode panel. Since there have been no experiments performed under defined light conditions before, the lamp was set on the highest possible power. Based on the previous preliminary experiments (Galica et al., 2021) (Hardy et al., 2019) the photolysis is believed to occur within couple of hours after the complex was exposed to the light of demanded wavelength. For this reason, the time schedule of the experiment was planned to focus on first two hours of exposition mainly. Within this time interval the reaction solution was sampled each 30 minutes until the time of exposition reached 2 hours. After reaching the two hours of exposition the sampling time was prolonged for 60 minutes and the reaction solution was sampled continually until we reached 7 hours of exposition. As negative controls two reaction mixtures measurements were performed. In the first case the reaction solution was exposed only to light of visible part of the diode spectra with a UV light source switched of. In the second case, the reaction solution was kept in the dark. The experimental set up is closely described in Tab.8.

Tab.8.: The experimental set up for measurement of reaction kinetics of 1026-Fe complex decomposition

Time of exposition [min]	Reaction mixtures		
	UV-Vis light	Vis- light	dark
0	T ₀ -UV	T ₀ -Vis	T ₀ -D
30	T ₁ -UV	T ₁ -Vis	T ₁ -D
60	T ₂ -UV	T ₂ -Vis	T ₂ -D
90	T ₃ -UV	T ₃ -Vis	T ₃ -D
120	T ₄ -UV	T ₄ -Vis	T ₄ -D
180	T ₅ -UV	T ₅ -Vis	T ₅ -D
240	T ₆ -UV	T ₆ -Vis	T ₆ -D
300	T ₇ -UV	T ₇ -Vis	T ₇ -D

The experiment was performed in two rounds when each experiment was analysed using a different analytical method. The samples collected during the first experiment

were analysed without any additional treatment using high performance liquid chromatography with Diode-Array Detection method (HPLC-DAD). The HPLC-DAD analysis was performed on Agilent 1260 Infinity II liquid chromatograph. Within this experiment significant problems with detection of the cyanochelin 1026 occurred and thus an optimisation of the analysis was performed (chapter 4.4.2). In the second experiment the samples from Vis and Dark reaction mixtures were excluded from the analysis based on the results from the first experiment. In this case the samples were analysed using HPLC-MS/MS method (details in chapter 4.3). Before the samples were submitted for the analysis, they were diluted with adequate volume of MeOH and basified to pH=10 using 1M NaOH according to the approach described in optimisation (chapter 4.4.2). The concentration of cyanochelin 1026 was derived from calibration curve, which was prepared from cyanochelin standard solution in concentration range 100-7,5 μM . The calibration standards were analysed in the same sequence as the samples were.

4.4.1.Determination of kinetic parameters of cyanochelin 1026-Fe complex decomposition under UV light exposition

An order of reaction of cyanochelin 1026- Fe^{3+} complex decomposition was determined using and integration method. The equations for reaction rates of each reaction order were transferred to their integration form. From the integration form an equation for reaction rate constant was expressed (see equations below). The reaction rate constant was subsequently calculated between initial concentration and concentration measured at time x (T_1 - T_7) using all possible formulas. The reaction order was then determined based on the variability between rate constants calculated by one formula.

Expression of reaction rate constant-zero order

$$\frac{d[A]}{dt} = -k$$

$$\int_{[A]_0}^{[A]_t} d[A] = -k \int_0^t dt$$

$$[A] = -kt + [A_0]$$

$$k = \frac{[A_0] - [A]}{(t_x - t_0)}$$

Expression of reaction rate constant-first order

$$\frac{d[A]}{dt} = -k[A]$$

$$\int_{[A]_0}^{[A]_t} \frac{1}{[A]} d[A] = -k \int_0^t dt$$

$$\ln \frac{[A]_t}{[A]_0} = -kt$$

$$k = \frac{\ln[A]_0 - \ln[A]}{(t_x - t_0)}$$

Expression of reaction rate constant-first order

$$\frac{d[A]}{dt} = -k[A]^2$$

$$\int_{[A]_0}^{[A]_t} \frac{1}{[A]^2} d[A] = -k \int_0^t dt$$

$$\frac{1}{[A]_t} - \frac{1}{[A]_0} = -kt$$

$$k = \frac{\left(\frac{1}{[A]_t} - \frac{1}{[A]_0} \right)}{(t_x - t_0)}$$

The reaction rate constant is a value, which is supposed to be always the same regardless the time of reaction. The reaction order is determined based on the variability among the values determine for various time points of the reaction. The determined values must be constant, when plotted against time. Thus, the calculated rate constants were plotted against the respective time. Subsequently the linear trend line was formed in each plot.

$$y = mx + c$$

m.....slope of the linear function

c.....y-intercept

The mathematical analysis of the results, which is describe above) revealed the photoreaction of cyanochelin 1026 to be reaction of zero order. Based on this finding

the half-time of photoreaction of cyanochelin 1026 was determined using the formula derived from integrated form of relation between reaction rate constant and concentration of reactant expressed for zero order reaction.

$$[A] = -kt + [A_0]$$

$$\frac{1}{2}[A_0] = [A_0] - kt_{\frac{1}{2}}$$

$$t_{\frac{1}{2}} = \frac{[A_0]}{2k}$$

4.5 Identification of photolytic fragments of cyanochelin m/z 1026-Fe³⁺ complex degradation with LC-MS

4.5.1. Identification of photolytic fragments of cyanochelin m/z 1026

During the photoreduction of Fe³⁺ ions, which is facilitated by cyanochelin 1026, the cyanochelin 1026 decomposed to various photolytic fragments. Two fragments, the fragments m/z 633 and m/z 634, have been already detected with the HPLC-MS/MS method during previous research on cyanochelin 1026 (Galica et al., 2021). In our case the detection of the photolytic compounds was performed in the same experiment as the determination of reaction kinetics of the cyanochelin 1026-Fe complex decomposition using the HPLC-MS/MS analytic method. The fragments were identified based on the comparison fragmentation spectra of tentative molecules and fragmentation spectrum of cyanochelin 1026.

4.6 Evaluation of stoichiometry of Fe³⁺ cations photoreduction facilitated by cyanochelin m/z 1026 and identification of photolytic activity of cyanochelin m/z 1026 photolytic fragments

The photodegradation of cyanochelin 1026, when exposed to the UV light in complex with Fe³⁺ results in formation of various photolytic fragments. In many cases the formed fragments keep the structural features required for chelation of Fe³⁺ cations-presence of such as one or two β-hydroxy aspartic acids in the fragment or other hydroxy groups. The presence of these moieties suggests possible further photolytic activity of these fragments towards the Fe³⁺ cations (Hardy et al., 2019) (Galica et al., 2021). Such a photolytic activity would lead to non-equimolar ratio between amount of cyanochelin

1026 amended to the reaction solution and amount of Fe^{2+} cations produced. Since there are more molecules in the reaction involved, it will be very difficult to describe the reaction stoichiometry through monitoring the concentration of Fe^{3+} chelators, as it was done within previous experiments. Thus, the stoichiometry of Fe^{3+} photoreduction was assessed through measurement of Fe^{2+} concentration, which is proportional to amount of chelators, which reacted with Fe^{3+} cations during the exposition to the UV light. According to the literature (Kreutzer et al., 2012) a photometric detection of Fe^{2+} in complex with bathophenanthroline disulfonic acid (BPDS) offers a convenient method for selective quantification of Fe^{2+} cations in water solutions. The BPDS is a chelator of divalent cations, which forms stable water soluble Fe^{2+} complexes with significant absorbance in 533 nm (Mudasir et al., 1998) (Hirayama et al., 2017).

4.6.1. Optimisation of Fe^{2+} detection with bathophenanthroline disulfonic acid (BPDS)

Before the stoichiometric experiments were performed, the BPDS detection method for Fe^{2+} cations was tested for two parameters to prove the reliability of the method. A first assessment of the BPDS detection method was finding an appropriate calibration standard, which possesses a good water solubility and stability in BPDS complex. For this purpose, three different substances were chosen, ferric nitrate standard in standard nitric acid (Sigma Aldrich, Fe con. 1000 mg l^{-1}), ferric chloride hexahydrate (Sigma Aldrich, 98%) and ferrous sulfate heptahydrate (Sigma Aldrich, $\geq 99\%$). The stock solutions of substances were prepared in 1 ml solutions with concentration 10 mM. Subsequently, 1 ml of working solutions were prepared by dilution of 40 μl of stock solutions with 860 μl dH_2O in fresh 1.5 ml Eppendorf tubes. Before addition of BPDS the Fe^{3+} cations were reduced to Fe^{2+} cations by addition of excessive amount of hydroxyl amine, an effective iron(III) reduction agent (Rao et al., 1959). The ferric nitrate and ferric chloride solutions were reduced by addition of 100 μl of 50% hydroxyl amine to the working solution. In the case of ferrous sulfate, the hydroxyl amine was replaced by the same dose of dH_2O . The concentration of ferrous cations in working solutions was 400 μM . In the following step an appropriate volume of buffered working solution was taken and diluted with 2mM MOPS buffer to obtain a 100 μl solution of a required concentration of ferrous cations. In this way several solutions of different concentrations were prepared. After mixing with MOPS the buffered solutions were diluted with 100 μl of the 1mM BPDS prepared from 1M bathophenanthroline disulfonic

acid (Sigma Aldrich, $\geq 98\%$) stock solution and the absorbance was measured at 535 nm using the Shimadzu UV-2700i spectrophotometer. The final concentrations of measured solution are presented in Tab.9. The concentration of solutions after amendment of BPDS correlates to the concentrations of calibration standards in kinetics experiments.

Tab.9.: Preparation of final ferrous solutions from working solutions by addition of MOPS buffer and BPDS colouring agent

Working solution concentration [μM]	Volume of working solution [μl]	Volume of MOPS buffer [μl]	Volume of BPDS [μl]	Final concentration [μM]
400	50	50	100	100
400	25	75	100	50
400	12,5	87,5	100	25
400	6,25	93,75	100	12,5
400	3,13	96,87	100	6,25
400	1,6	98,4	100	3,125
400	0,8	99,2	100	1,56

As a second parameter the UV stability of BPDS-Fe²⁺ complex was tested. The BPDS complex was prepared in 1,5 ml aliquot in 2 ml Eppendorf tube from 1M bathophenanthroline disulfonic acid (Sigma Aldrich, $\geq 98\%$) stock solution, 10M ferrous sulfate heptahydrate (Sigma Aldrich, $\geq 99.0\%$) stock solution and 2 mM MOPS buffer. The final concentrations of ferrous sulfate and BPDS were 0,05 mM and 200 μM, respectively. The Eppendorf tube with BPDS-Fe²⁺ solution was exposed to UV-Vis light as in the kinetics experiment (chapter 4.3.1.). Based on the results from kinetics experiment the samples were collected in 30 minutes time intervals. The absorbance of the samples was measured at 535 nm using the Shimadzu UV-2700i spectrophotometer.

4.6.2. Evaluation of stoichiometry of photoreduction of Fe³⁺ cations facilitated by cyanochelin m/z 1026

The stoichiometric experiment was performed based on the information obtained during kinetics experiment. The experiment was designed in three reaction mixtures, two

experimental reaction mixture and a negative control reaction mixture. In experimental reaction mixtures both reaction components were present, however, in the case of first reaction mixture the BPDS was amended before the UV light exposition, while in the second reaction mixture the BPDS was added after the exposition to UV light. In the case of negative control cyanochelin 1026 was replaced by dH₂O prepared equally to the other reaction mixtures. Within this experiment 50 µM solution of siderophore was amended with various concentrations of Fe³⁺ cations and exposed to the UV-Vis light for four hours. The reaction mix was prepared in 0,35ml aliquots in 1,5 ml Eppendorf tubes. The Fe³⁺ cations were provided from 10 mM stock solution of Fe(NO₃)₃ (details in chapter 4.3.1). The cyanochelin 1026 was provided from 10 mM stock solution prepared the isolated compound. The 1mM solution of BPDS was added to the reaction solution at respective time. The reaction solutions were prepared according to the Tab.10. The concentration of Fe²⁺ produced in the reaction was determined using an external calibration curve. The calibration curve was prepared in 350 from iron (II) sulfate in range 12,5-150 µM. The calibration standards were prepared in 0,35ml aliquots in 1,5 ml Eppendorf tubes from freshly prepared 10 M stock solution of iron (II) sulfate by dilution with dH₂O to the adequate concentrations. The absorbance of the samples was measured at 535 nm using the Shimadzu UV-2700i spectrophotometer.

Tab.10.: Preparation of reaction solution from stock solutions of reactants in dH₂O and subsequent addition of colouring agent BPDS

cyan.1026[µM]	c Fe³⁺ [µM]	V1026 stock [µl]	V Fe³⁺ stock [µl]	V dH₂O stock [µl]	V BPDS [µl]
50	25	3,5	1,75	344,75	350
50	50	3,5	3,5	342	350
50	75	3,5	5,25	341,25	350
50	100	3,5	7	339,5	350
50	125	3,5	8,75	337,75	350
50	150	3,5	10,5	336	350
50	200	3,5	14	332,5	350

4.6.3. Analysis of Fe^{2+} detection problems from stoichiometric experiment

Within a stoichiometric experiment Fe^{2+} cations were detected with BPDS. In the case of second experimental reaction mixture, we have detected very low concentrations of Fe^{2+} , which does not correlate with kinetics experiment. Based on these results the stability of free Fe^{2+} cations in MOPS buffer were tested upon an oxygenic and anoxygenic atmosphere. According to the literature the Fe^{2+} cations are vulnerable to re-oxidation to Fe^{3+} in the presence of atmospheric oxygen (B. Morgan et al., 2007). Thus 500 μl of 100 μM Fe^{2+} solution in MOPS buffer (2 mM) was prepared from 10 mM ferrous sulphate stock solution in two reaction mixtures in 1,5 ml Eppendorf tubes in triplicates. In the case of first reaction mixture the Fe^{2+} solution was prepared without any additional treatment for oxygen elimination. The second reaction mixture was treated with N_2 flux for 3 hours to eliminate oxygen from the solution. Subsequently both reaction mixtures were exposed to the UV-Vis light for four hours. After the exposition 500 μl of 1 mM BPDS solution was amended and the absorbance was measured at 535 nm using the Shimadzu UV-2700i spectrophotometer.

The stability of the free Fe^{2+} cations was also tested for vulnerability to buffer presence. For this assessment 500 μl of 100 μM Fe^{2+} solution was prepared in two reaction mixtures in 1,5 ml Eppendorf tubes in triplicates. In the case of first reaction mixture the Fe^{2+} solution was prepared in 2 mM MOPS buffer, while the second reaction mixture was prepared in dH_2O . Subsequently both reaction mixtures were exposed to the UV-Vis light for four hours. After the exposition 500 μl of 1 mM BPDS solution was amended and the absorbance was measured at 535 nm using the Shimadzu UV-2700i spectrophotometer.

5. Results

5.1 Isolation of cyanochelin 1026

5.1.1. Optimisation of cyanochelin m/z 1026 extraction from dried biomass

Two solvents were tested in different volumetric ratios to obtain the most suitable solvent for cyanochelin m/z 1026 extraction from dried biomass. The extraction yield was evaluated as peak areas obtained from HPLC-MS/MS analysis. The results from extraction with methanol (MeOH) solvents showed that extraction efficiency increases proportionally with increasing MeOH content (Fig.14.). The highest yield was observed in the case of first extraction step using 100% MeOH, and the following second and third extraction steps showed a significant drop in amount of extracted cyanochelin suggesting nearly quantitative extraction of the compound. Different results were observed in the case of 80% MeOH and 60% MeOH solvents. The 80% MeOH extraction showed only a minor decline in amount of extracted cyanochelin in the first step when compared to the 100% MeOH and the second extraction step provided almost equal amount. In the case of 60% MeOH, the second extraction exceeded the first extraction step. Thus, these two solvents were considered appropriate MeOH based solvents for cyanochelin 1026 extraction assuring higher yields compare to other MeOH mixtures.

The results from extraction using acetonitrile (ACN) based solvents has showed a comparable yield of extracted cyanochelin 1026. The highest yield was observed in 80% ACN, 60% ACN and 40% ACN (Fig.15.). Most efficient first extraction step was measured for 60% ACN solvent (peak area 10×10^7). In the case of second extraction step, the almost same yield was observed in three most efficient solvents. In 80% ACN solvent, the compound yield in the second and third even exceeded the yield from the first extraction, while the solvents with composition of 60, 40 and 20 % ACN showed a decrease in amount of extracted cyanochelin between first, second and third extraction step. Based on these results, the 60% ACN solvent was considered as the most suitable ACN based solvent for cyanochelin extraction.

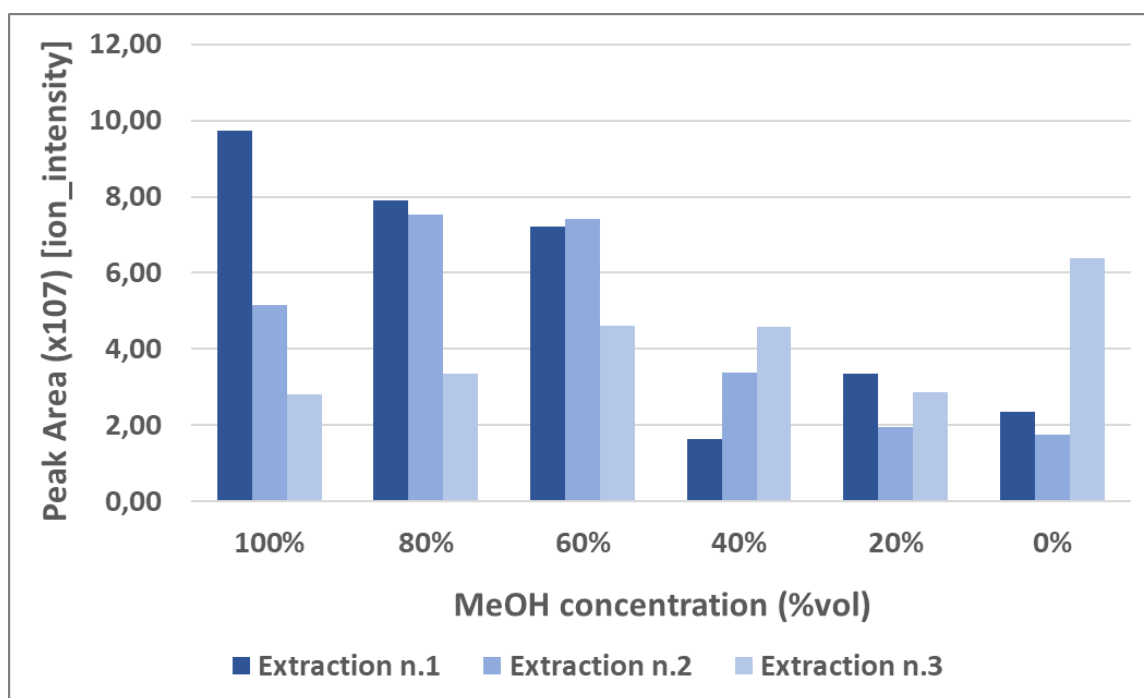


Fig.14.: Peak areas of cyanocheilin 1026 in HPLC-MS chromatogram (extracted ion chromatogram) resulting from analyses of samples obtained using various MeOH concentrations

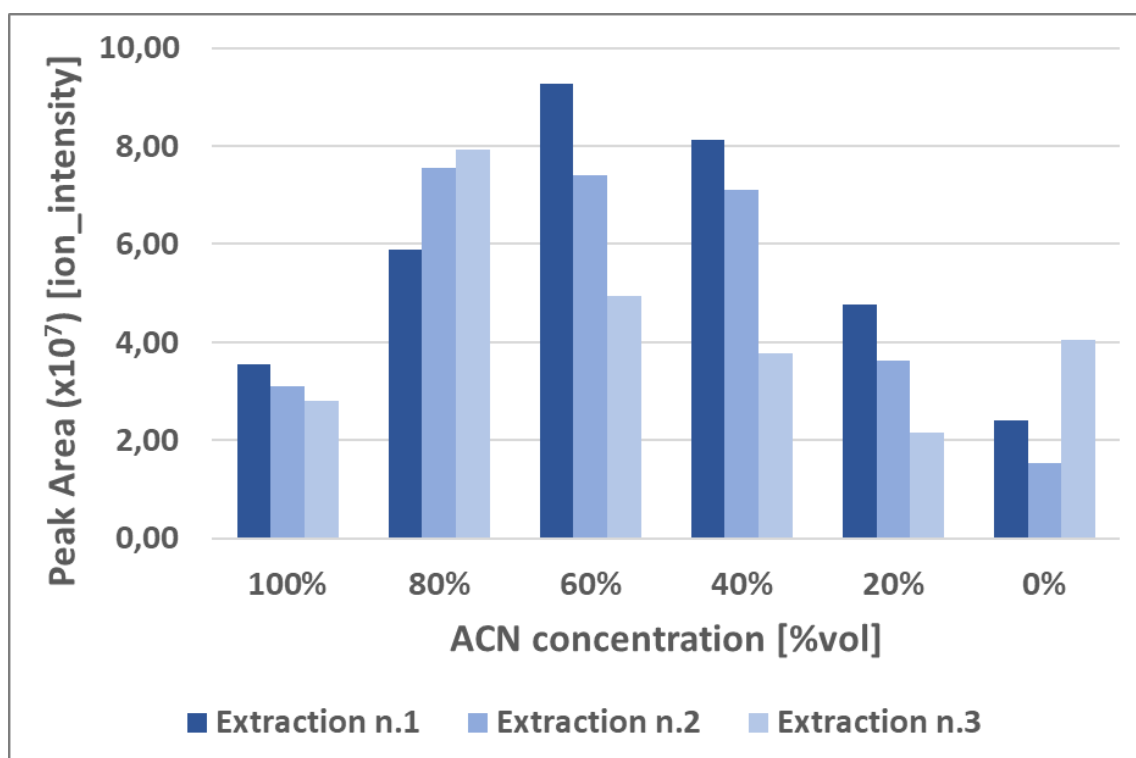


Fig.15.: Peak areas of cyanocheilin 1026 in HPLC-MS chromatogram (extracter ion chromatogram) resulting from analyses of samples obtained using various ACN concentrations

When compared the most suitable extraction solvents from both experiment- 80% MeOH, 60% MeOH and 60% ACN, the 60% ACN solvent gives the highest overall yield of cyanochelin. Thus, this solvent was utilized for all the following extraction procedures.

5.1.2. Isolation of cyanochelin m/z 1026 with preparative HPLC

Several successful isolations of cyanochelin m/z 1026 with sufficient yield and suitable purity were performed. The amount of processed dried biomass, the amount of isolated cyanochelins as well as a total yield are shown in Tab.11. A part of the isolated cyanochelin 1026 (10 mg) was sent to Dr. Valeria Costantino PhD. from “TheBlueChemistryLab”, Department of Pharmacy, University of Naples Federico II, Napoli, Italy, where the NMR structural analysis of cyanochelin 1026 molecule was performed. The NMR analysis elaborated the structural details of N-terminal part and revealed the presence of two hydroxyl groups at β and γ positions of the fatty acid chain as well as methyl group on beta position and on the thiazole group, which were previously unavailable (Fig.16).

Tab.11.: Summarization of yields from isolation of cyanochelin 1026

Date of finishing the isolation	Dry biomass weight [g]	Crude extract volume [ml]	Concentrated extract volume [ml]	Cyanochelin m/z 1026 yield [mg]
10.2.2021	0,618	93	3,6	2
3.8. 2021	3,384	526	26,4	15
7.4.2022	3,770	567	28,9	16
12.5.2022	3,436	510	25,4	30
21.6.2022	3,404	495	24,1	17
Total mass yield				80

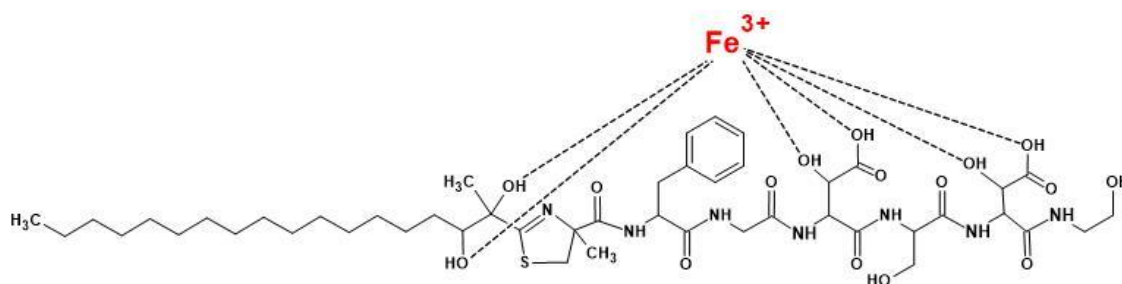


Fig.16.: Chemical structure of cyanochelin 1026 structure and proposed coordination of Fe³⁺ cation via β-hydroxy aspartate groups (known) and hydroxyl groups located on fatty acid chain (proposed)

5.2 Determination of reaction kinetics of cyanochelin m/z 1026-Fe³⁺ complex decomposition upon exposition of UV-light

5.2.1. Optimisation of cyanochelin 1026 detection

The optimisation of the HPLC-DAD method was performed to ensure the reliable quantification of cyanochelin in the reaction solution. After the detection method was established including the selection of proper stationary phase (chapter 4.4.2), the photolysis was performed. The calibration curve was created from calibration standard cyanochelin solutions in range 6,5-150 μM. The linear equation of calibration curve and coefficient of reliability were determined to be $y = 6,1015x + 6,9873$ (Fig.17).

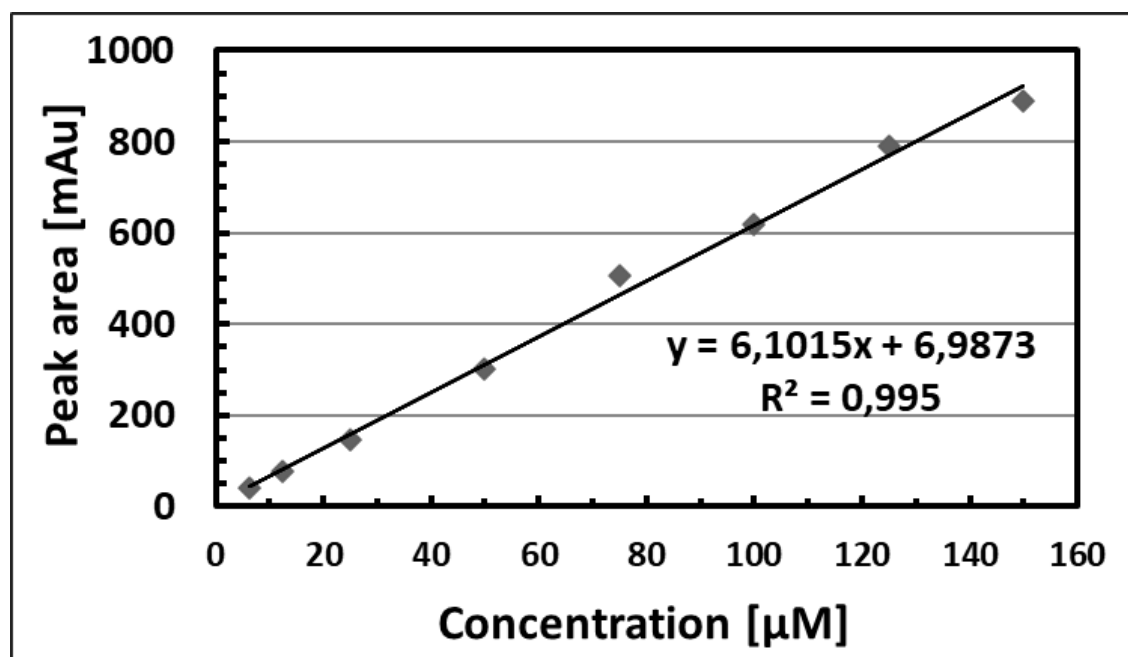


Fig.17.: Calibration curve of cyanochelin 1026 during optimisation experiment.

The photolytic experiment was performed in three reaction mixtures, which were analysed sequentially in following order-UV-Vis, Vis and dark. The peak areas measured from the HPLC chromatograms ($\lambda=254$ nm) are shown in Tab.S1. The concentration of the cyanochelin was determined from linear relation between cyanochelin concentration and peak area, which was derived from the calibration equation.

$$y = 6,1015x + 6,9873$$

$$x = \frac{y - 6,9873}{6,1015}$$

x..... cyanochelin concentration [μM]

y.....peak area

The development of cyanochelin concentration in time in analysed samples is depicted on Fig.18. In the case of the Vis and Dark reaction mixtures results have showed no significant changes in cyanochelin concentration in time, although the detected concentration corresponds to half concentration ($\sim 50 \mu\text{M}$) of the compound used in the experiment ($\sim 100 \mu\text{M}$). In the case of UV-Vis reaction mixture a unexpected trend was observed at the beginning of the experiment, when the concentration of cyanochelin 1026 was increasing in time in UV-Vis reaction mixture, which conflicts the theory. Subsequently a significant concentration drop was observed between the second and the third hour of exposition. Based on this finding a further optimisation was performed.

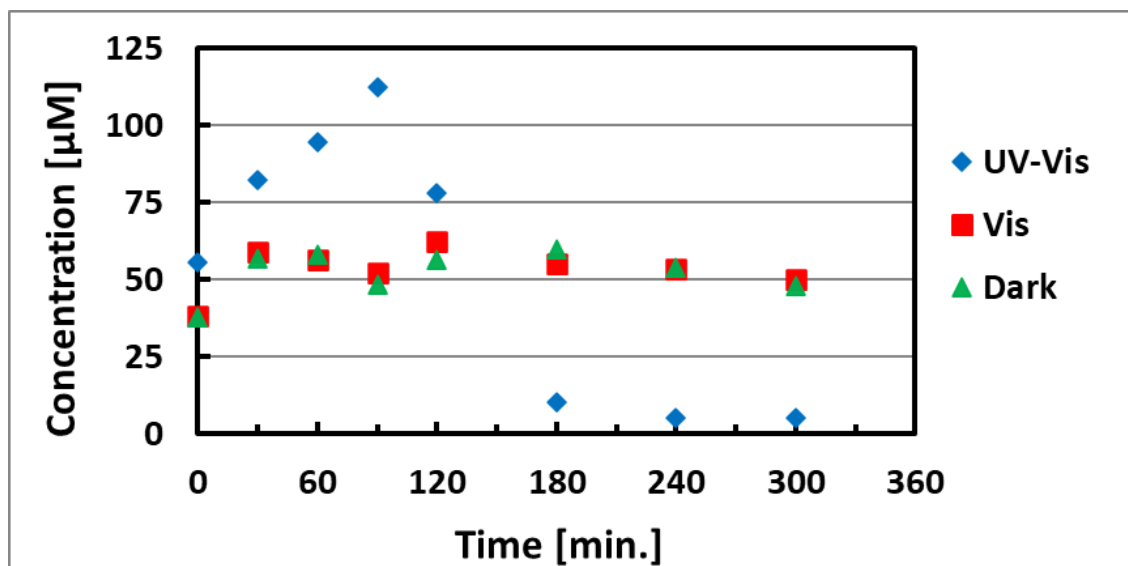


Fig.18.: The development of cyanochelin 1026 concentration in time upon exposition to three various light conditions- UV-Vis, Vis and dark measured within the optimisation experiment derived from measurement of absorbance at 254 nm.

Based on the previous result it can be concluded that significant proportion of the analyte has been lost during the procedure. Therefore, next step of optimisation was focused on the reaction solution. The solubility of cyanochelin and cyanochelin-Fe³⁺ complex was tested at neutral (pH=7) and basic (pH=10) conditions and with amendment of MeOH in various doses using HPLC-DAD analytical method. The concentration of cyanochelin in the samples was calculated from formula derived from calibration curve equation; $y=6,5743x-4,7667$ (Fig.19).

$$y = 6,5743x - 4,7667$$

$$x = \frac{y + 4,7667}{6,5743}$$

x..... cyanochelin concentration [µM]

y.....peak area

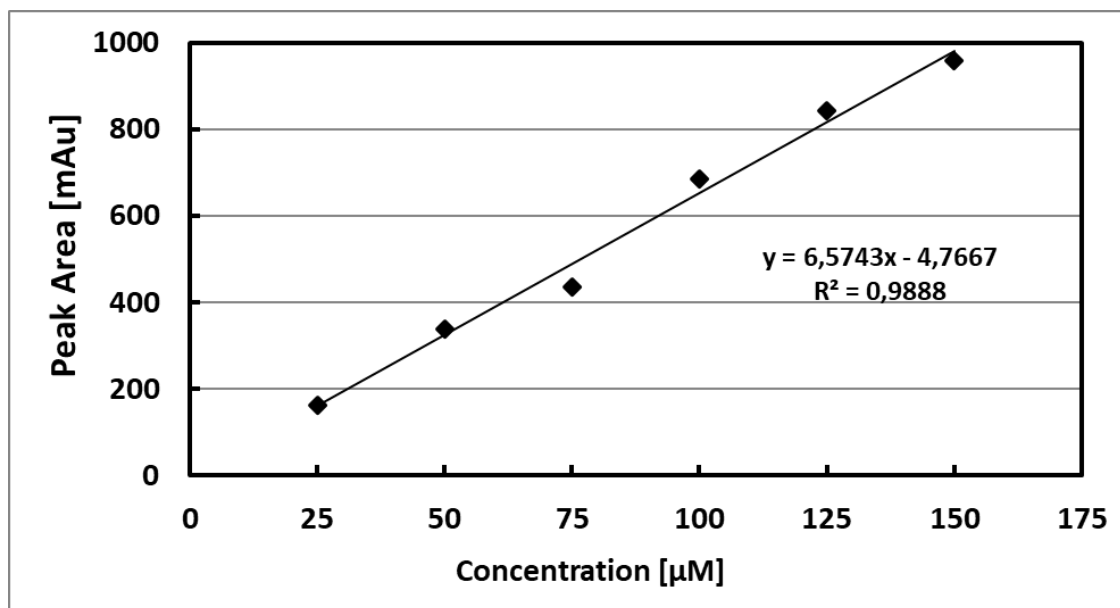


Fig.19.: Calibration curve of cyanochelin 1026 concentration during solubility optimisation experiment with peak area measured as absorbance integral (mAu)

The concentrations of cyanochelin measured for samples with neutral pH exhibited significant differences among individual samples with different concentration of MeOH and in general the analysis of samples exhibits a poor recovery of the analyte (Fig.20). The 50 µM concentration, which was established in the solution before the analysis was detected only in the case of non-complexed cyanochelin 1026 in control sample prepared in MeOH. In the rest of the samples the concentration was detected at approximately one half on initial value. In the case of complexed cyanochelin 1026 the 50 µM was detected in the case of samples containing either 0% MeOH or 20% MeOH. The rest of the samples has shown poor detection as well. A better recovery of the analyte in general was found in the case of samples prepared with basic pH (Fig.21). In this case the original concentration 50 µM was detected in most of the non-complexed samples except 0% MeOH and 40% MeOH. The complexed samples showed even better results, when the initial concentration was detected at all MeOH concentrations. Based on these results the basic 50% MeOH solution was established as the most suitable solution for HPLC analysis of complexed cyanochelin. These results suggest that samples treatment with MeOH and NaOH is necessary to assure proper cyanochelin quantification using HPLC analysis.

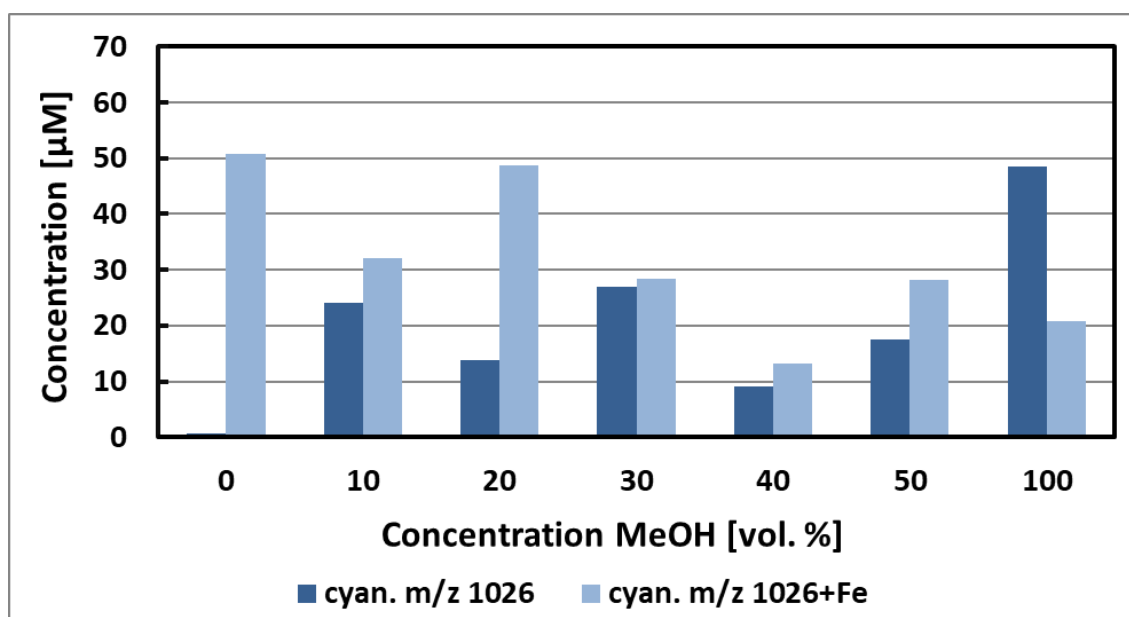


Fig.20.: Concentration of cyanochelin 1026 measured in solutions with various methanol concentration at neutral pH

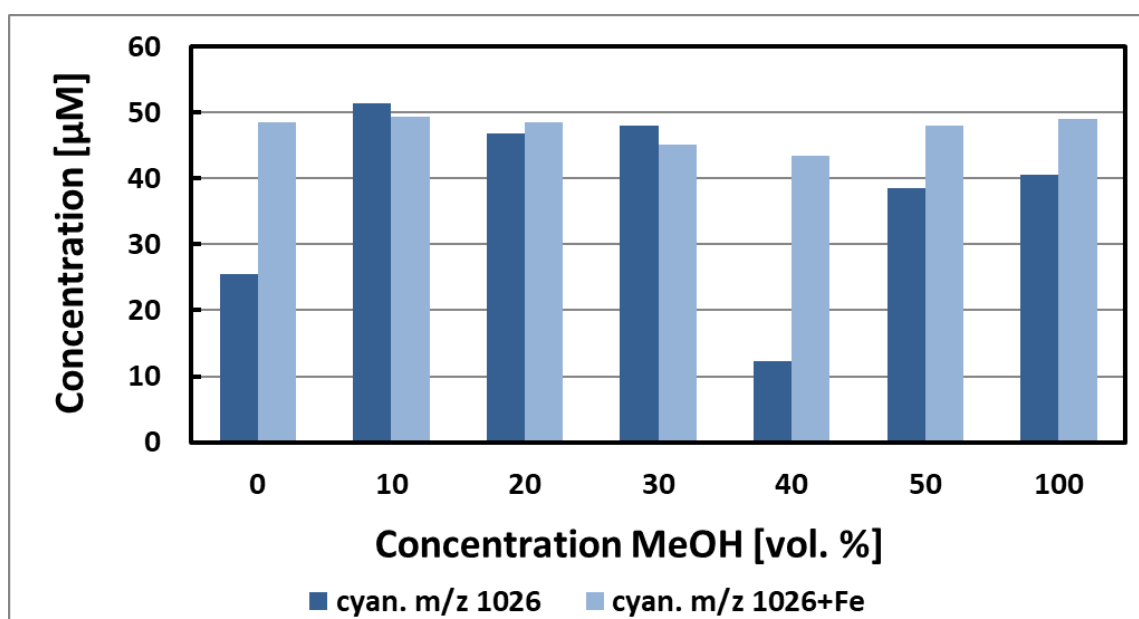


Fig.21.: Concentration of cyanochelin 1026 measured in solutions with various methanol concentration at basic pH

5.2.2.Determination of reaction kinetics of cyanochelin 1026-Fe complex decomposition under UV light exposition

The analysis of samples from photolytic experiment was further performed using HPLC-MS/MS and sample preparation described above. The compound of interest was

quantified with integration of extracted ion chromatograph of cyanochelin ion $[M+H]^+=1026$. The results showed a gradual decline in concentration of cyanochelin 1026 in time (Fig.23). At the beginning of the photoreaction the concentration of cyanochelin was determined to be $c=52,4 \mu\text{M}$, which correspond to the concentration established in reaction mixture. During the photoreaction cyanochelin concentration was gradually decreasing through the time of exposition up to T_6 (240 min.), when the concentration reached zero value and did not change within the last 60 minutes of exposition (T_7 -300 min.). The concentration of cyanochelin was calculated from the formula derived from calibration curve equation; $y=0,0571x+0,2989$ (Fig.22). The calculated concentrations of cyanochelin measured at each time point of the experiment are summarized in Tab.S2.

$$y = 0,0571x + 0,2989$$

$$x = \frac{y - 0,2989}{0,0571}$$

x..... cyanochelin concentration [μM]

y.....peak area

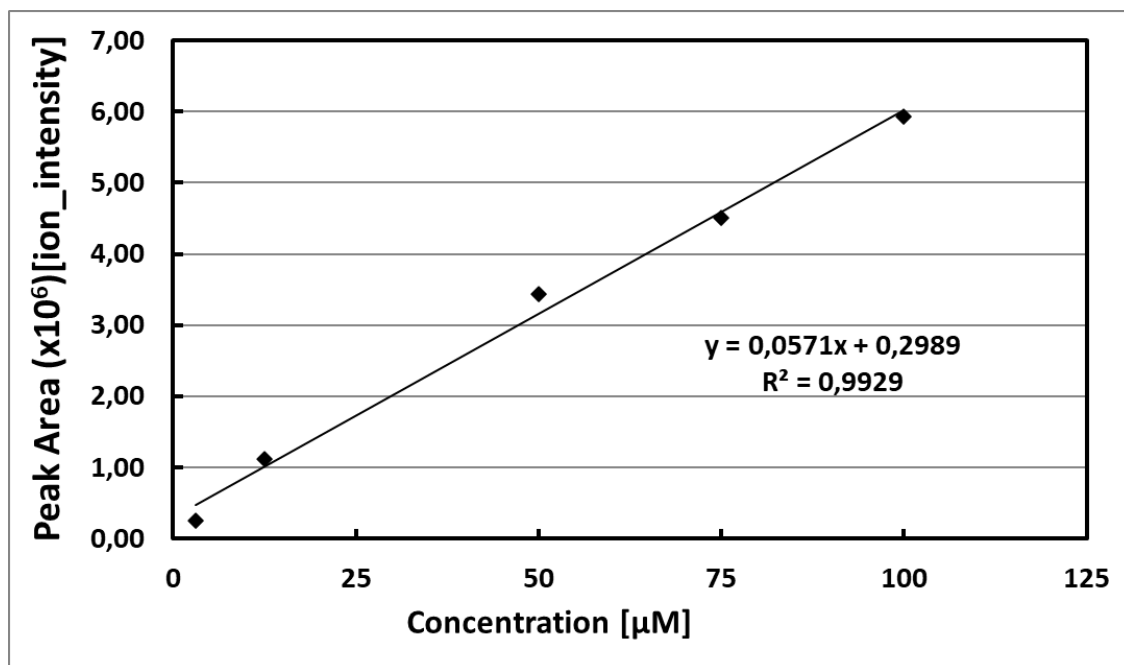


Fig.22.: Calibration curve for determination of cyanochelin 1026 concertation in reaction kinetics experiment.

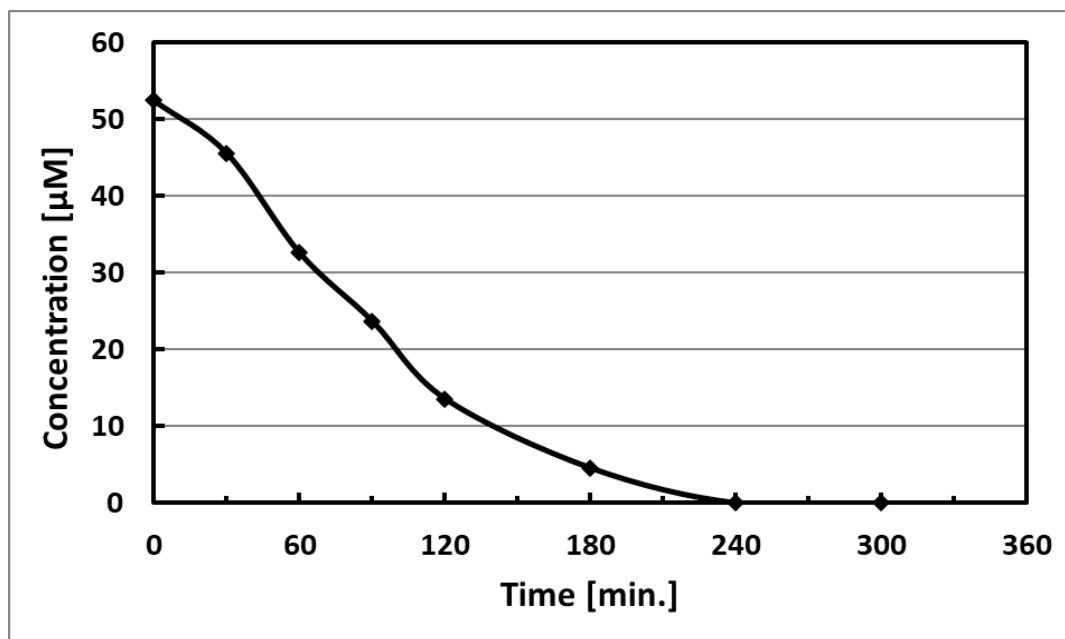


Fig.23.: The development of cyanochelin 1026 concentration in reaction solution in time upon exposition to UV-Vis light

The order of the reaction was determined based on the concentrations of cyanochelin measured during the photoreaction via integration method. The reaction constants were calculated using three various formulas each representing one type of reaction order (see chapter 4.4.1). Before plotting the reaction constants to the diagram against time, the values for zero, first and second reaction order were multiplied by 10^3 , 10^3 and 10^6 respectively (Tab.S2.). From the respective graphs of edited rate constants against time the smallest slope, $m=1,51 \cdot 10^{-3}$, of the trend line was observed in the case of zero order reaction (Fig.24), which implies that the reaction proceeds in kinetics of zero order with reaction rate constant $k=4,89 \times 10^{-3} \mu\text{M} \cdot \text{s}^{-1}$. The slopes of linear trend line in the case of first and second reaction order were determined to be $m=5,72 \cdot 10^{-3}$ (Fig.25) and $m=1,12 \cdot 10^{-1}$ (Fig.26) respectively. The half-time of the reaction was calculated to be $t_{1/2}=5,32 \times 10^3 \text{ s}$ ($\approx 89 \text{ min.}$) using the respective formula for reaction half-time for zero order reaction (see chapter 4.4.1).

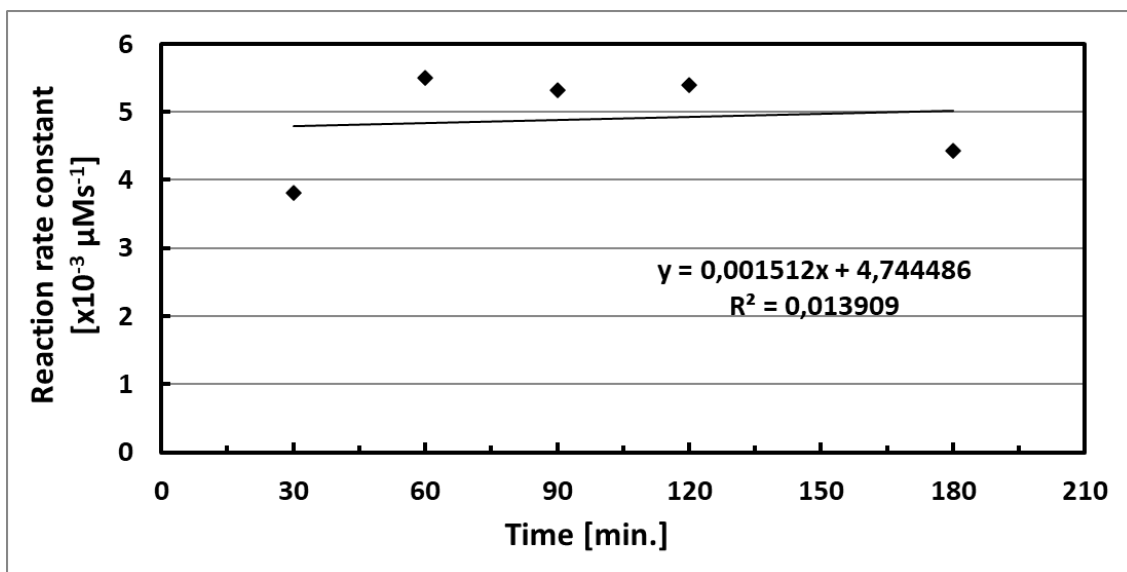


Fig.24.: The reaction rate values calculated for respective time points of the photolytic degradation of cyanochelin 1026 according to the formula for zero order reaction

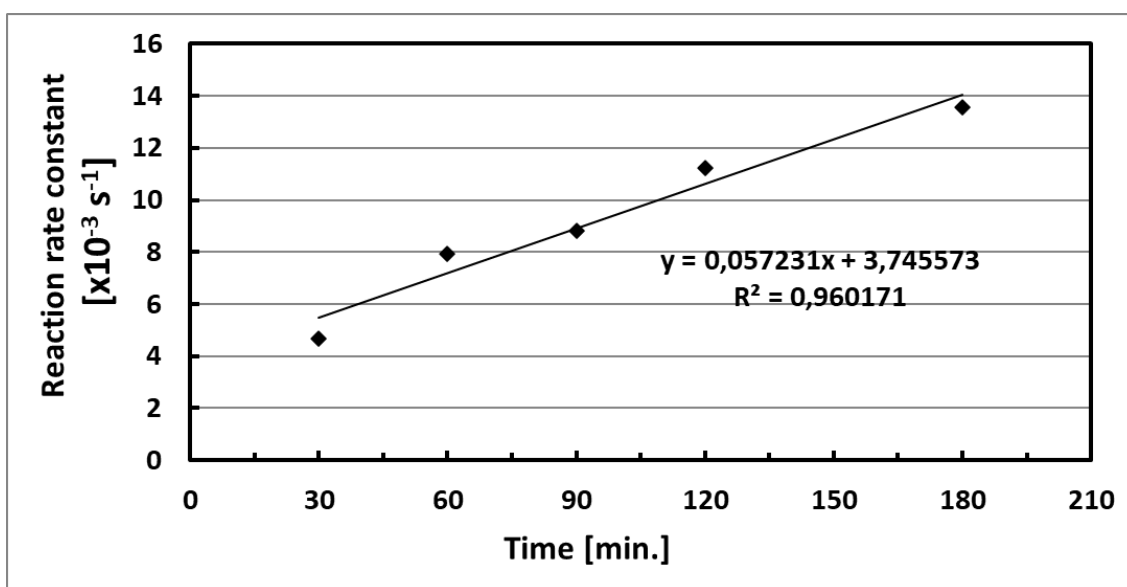


Fig.25.: The reaction rate values calculated for respective time points of the photolytic degradation of cyanochelin 1026 according to the formula for first order reaction

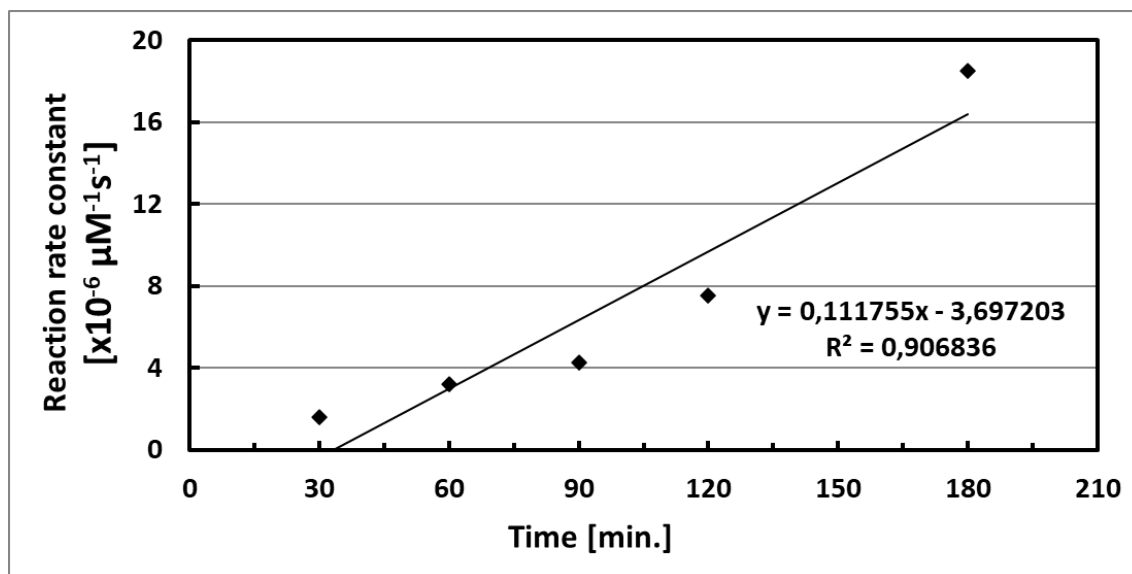


Fig.26.: The reaction rate values calculated for respective time points of the photolytic degradation of cyanochelin 1026 according to the formula for second order reaction

5.3 Identification of photolytic fragments of cyanochelin m/z 1026-Fe³⁺ complex degradation with HPLC-MS/MS

The photolytic products of cyanochelin 1026 were identified using the HPLC-MS/MS analysis. The identification based on the comparison of fragmentation spectra of cyanochelin 1026 (Fig.27) and tentative molecules has revealed four photolytic products of cyanochelin 1026 with respective mass to charge ratio: 633 m/z, 634 m/z, 851 m/z and 980 m/z. The fragmentation spectra of these compounds are shown in Fig.29, Fig.30, Fig.31 and Fig.32 respectively. The comparison of fragmentation spectra of tentative molecules with cyanochelin 1026 fragmentation spectrum has identified three signature ions, 144,04 m/z, 291,11 m/z and 319,10 m/z, which can be found in fragmentation spectra of both the maternal molecule of cyanochelin 1026 and all identified tentative photoproducts. The structure of these signature ions corresponds to the fragments a1-FA, a2-FA and b2-FA respectively, which are shown on Fig.28. In the case of 633m/z and 634 m/z there has been not another conformity with cyanochelin 1026 fragmentation spectrum observed. The comparison of these two fragmentation spectra has revealed that the measured spectra are almost identical and thus a high structural similarity is expected. The structure of these two fragments has been already revealed in previous study focused on cyanochelin 1026 (Galica et al., 2021) and corresponds to the cleavage of the peptide bond close between $\beta\text{OH-Asp}$ and Ser residues (ion b4, Fig. 27).

In the fragmentation spectrum of tentative molecules 851 m/z and 980 m/z additional signature ions 376,13 m/z, 507,15 m/z and 531,36 m/z were detected, which can be found also in the fragmentation spectra of cyanochelin 1026. These two signature ions correspond structurally to the cyanochelin 1026 fragments b3-FA, b4-FA and a2 respectively, which are showed on Fig. 28.

Based on the knowledge of structure of signature ions and molecular mass, the structures of tentative molecules were estimated and drawn in Chemskech software. In the case of 851 m/z the signature ion 507,15 m/z indicates that the cleavage of cyanochelin 1026 occurred at peptide chain in location from serine to the C-terminus. The signature ion 531,36 m/z then confirms the presence of fatty acid moiety in the molecule of tentative molecule 851 m/z. The final structure of tentative molecule 851 m/z was derive from the difference between molecular mass of cyanochelin 1026 and 851 m/z (175 Da), which corresponds to the mass of [β -OH-Asp]-ethanolamine dipeptide fragment (Fig.33). The structure of tentative molecule 980 m/z was revealed in the identical way as for 851 m/z. The difference between molecular mass of cyanochelin 1026 and 980 m/z (46 Da) was identified to correspond to the molecular mass of CO₂ and two hydrogen atoms, which cleaved from the β -hydroxy aspartic acid (Fig.33).

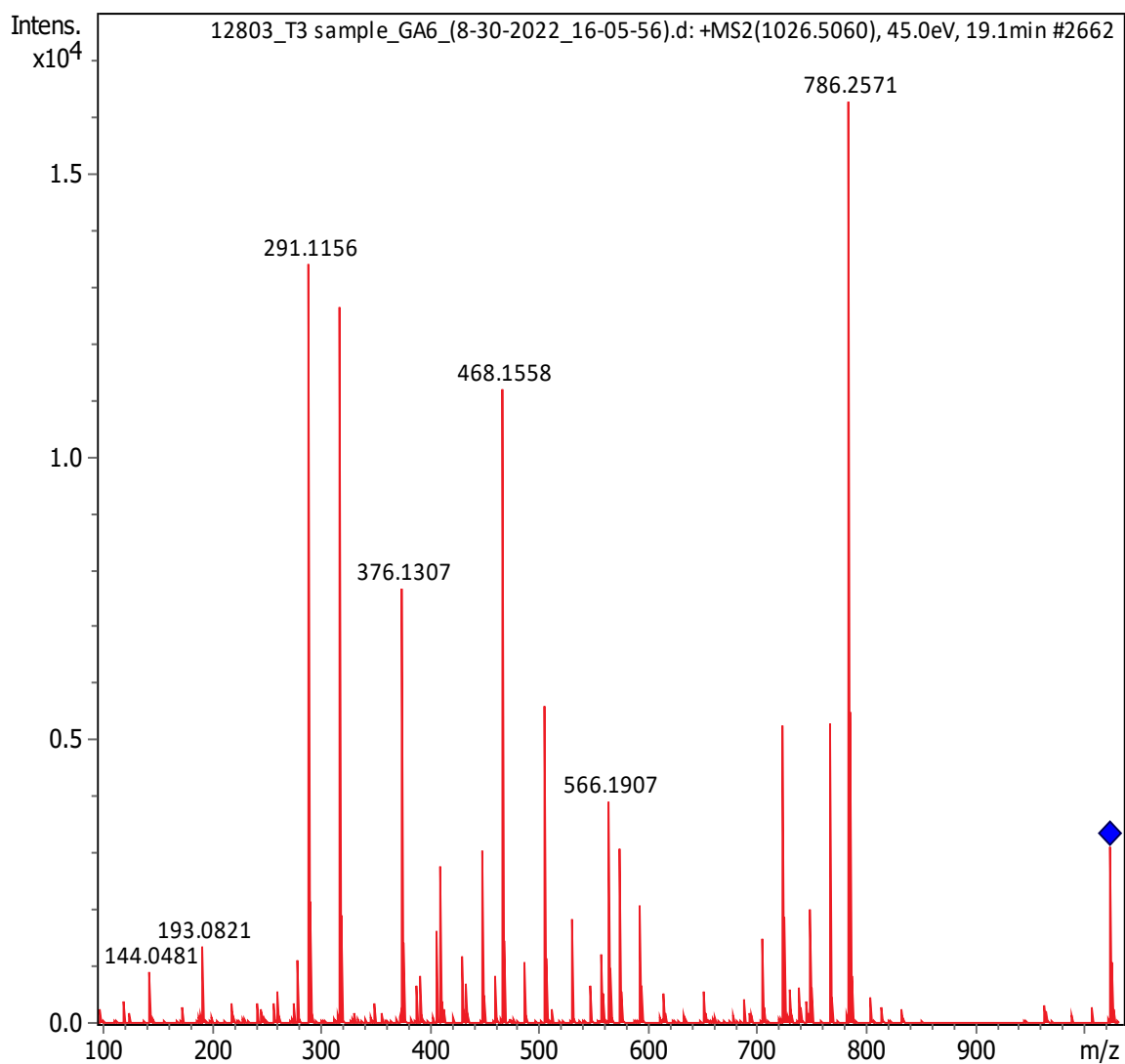


Fig.27.: Fragmentation spectra of cyanochelin 1026 obtained from the HPLC-MS/MS analysis of T₃ sample during the reaction kinetics experiment.

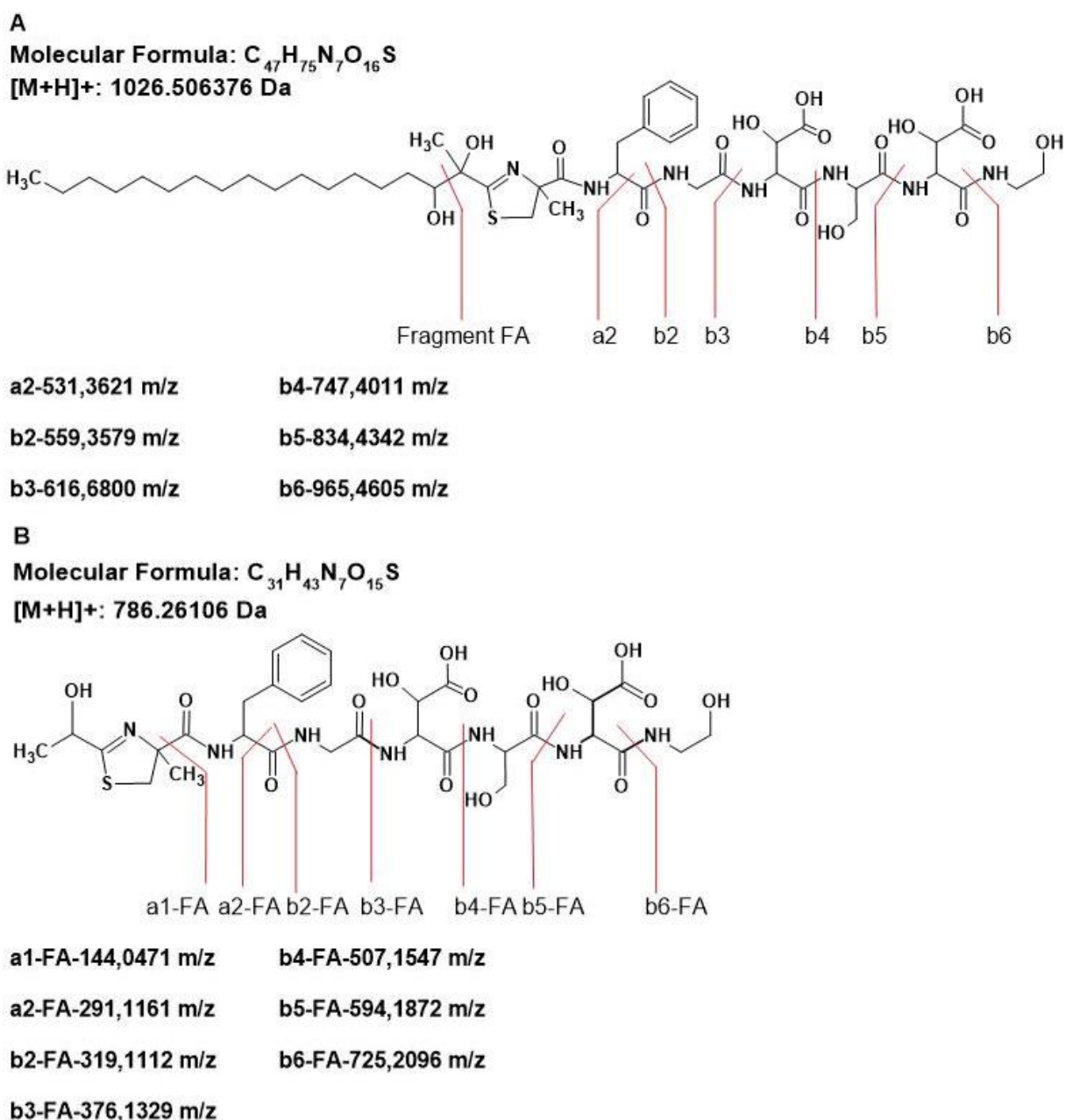


Fig.28.: The cleavage of cyanochelin 1026 molecule to respective fragments during the fragmentation of the molecule in MS/MS analysis. A- molecule of cyanochelin 1026 and positions of cleavage sites suggested based on the masses observed in fragmentation spectra of cyanochelin 1026. B- molecule of cyanochelin after cleavage of fatty acid moiety and respective positions of cleavage sites suggested based on the masses observed in fragmentation spectra of cyanochelin 1026. Abbreviations: FA-fatty acid, a2,b2-6- fragment of cyanochelin 1026, a2-FA,b2-7-FA- fragments of cyanochelin 1026 after cleavage of fatty acid moiety

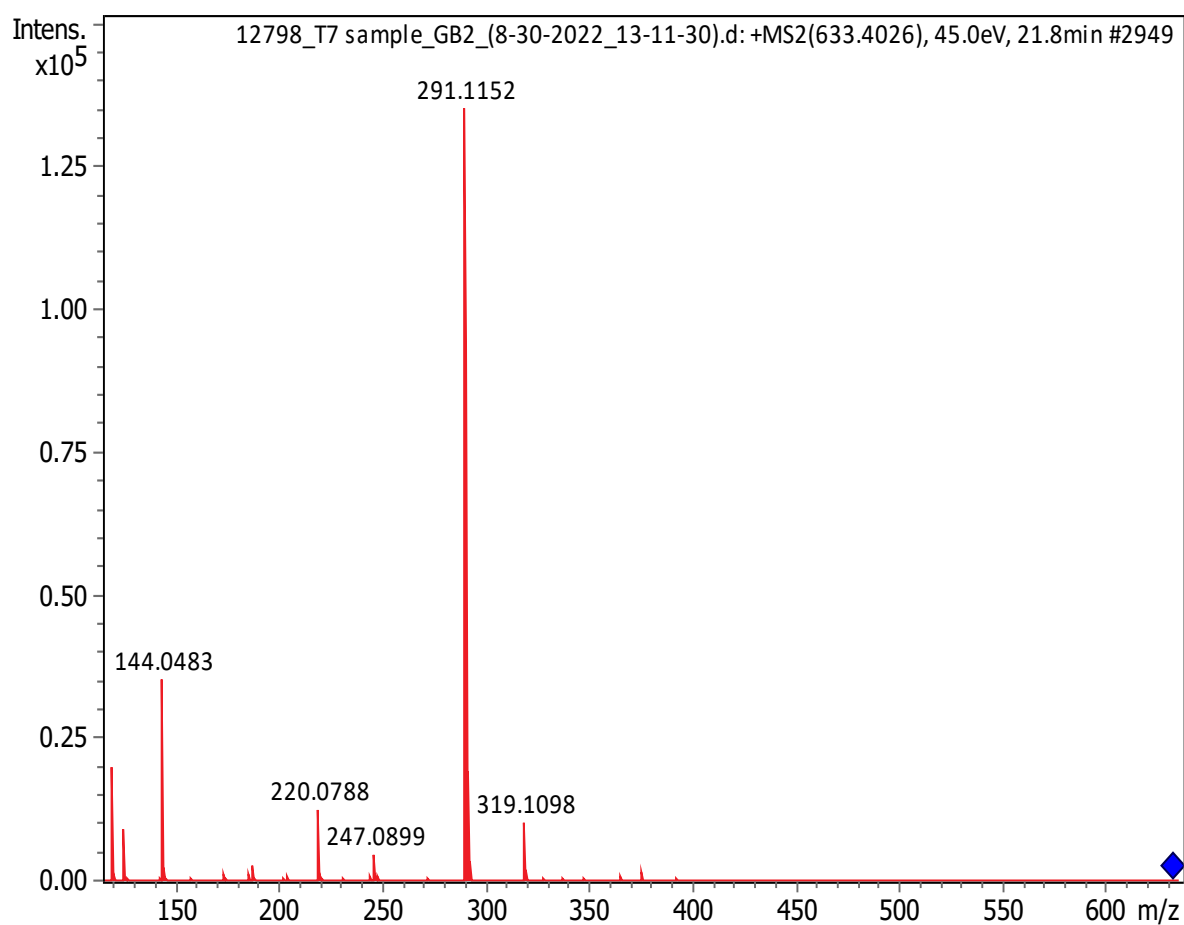


Fig.29.: Fragmentation spectra of photolytic fragment 633 m/z obtained from the HPLC-MS/MS analysis of T₇ sample during the reaction kinetics experiment.

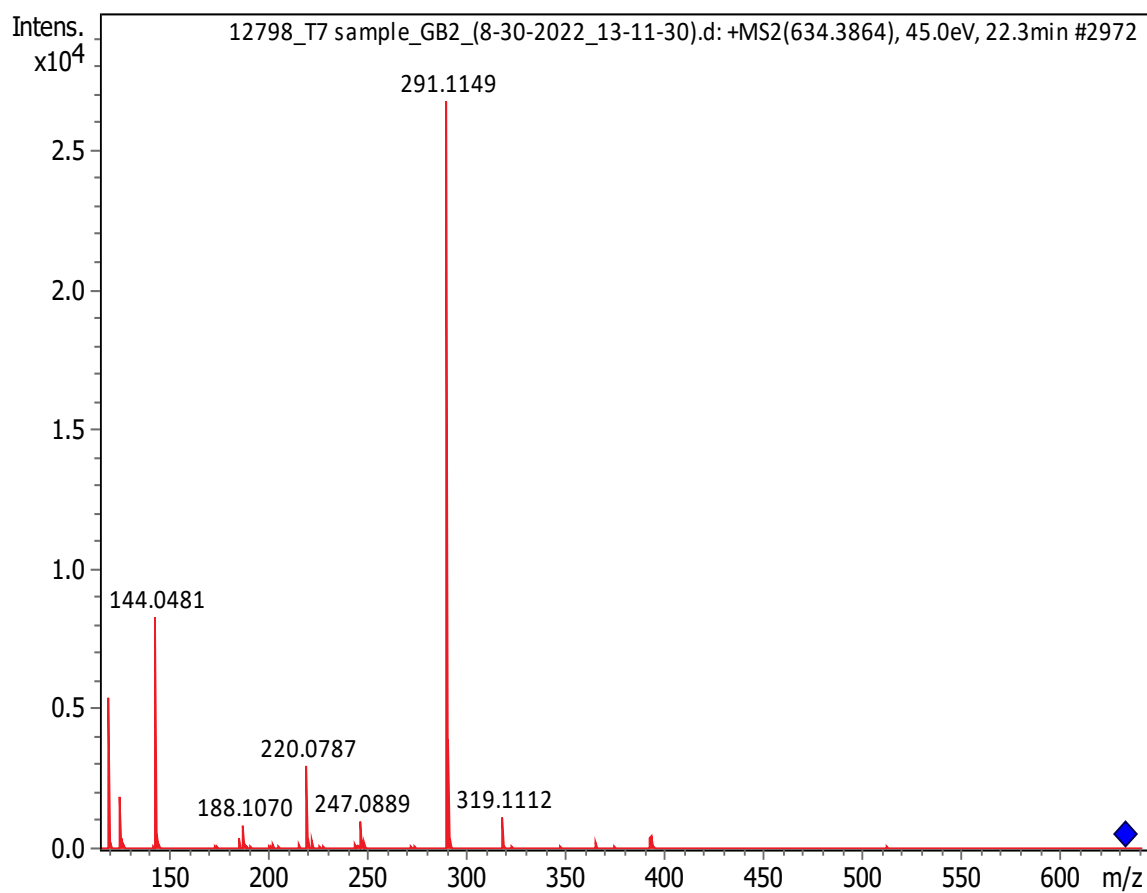


Fig.30.: Fragmentation spectra of photolytic fragment 634 m/z obtained from the HPLC-MS/MS analysis of T_7 sample during the reaction kinetics experiment.

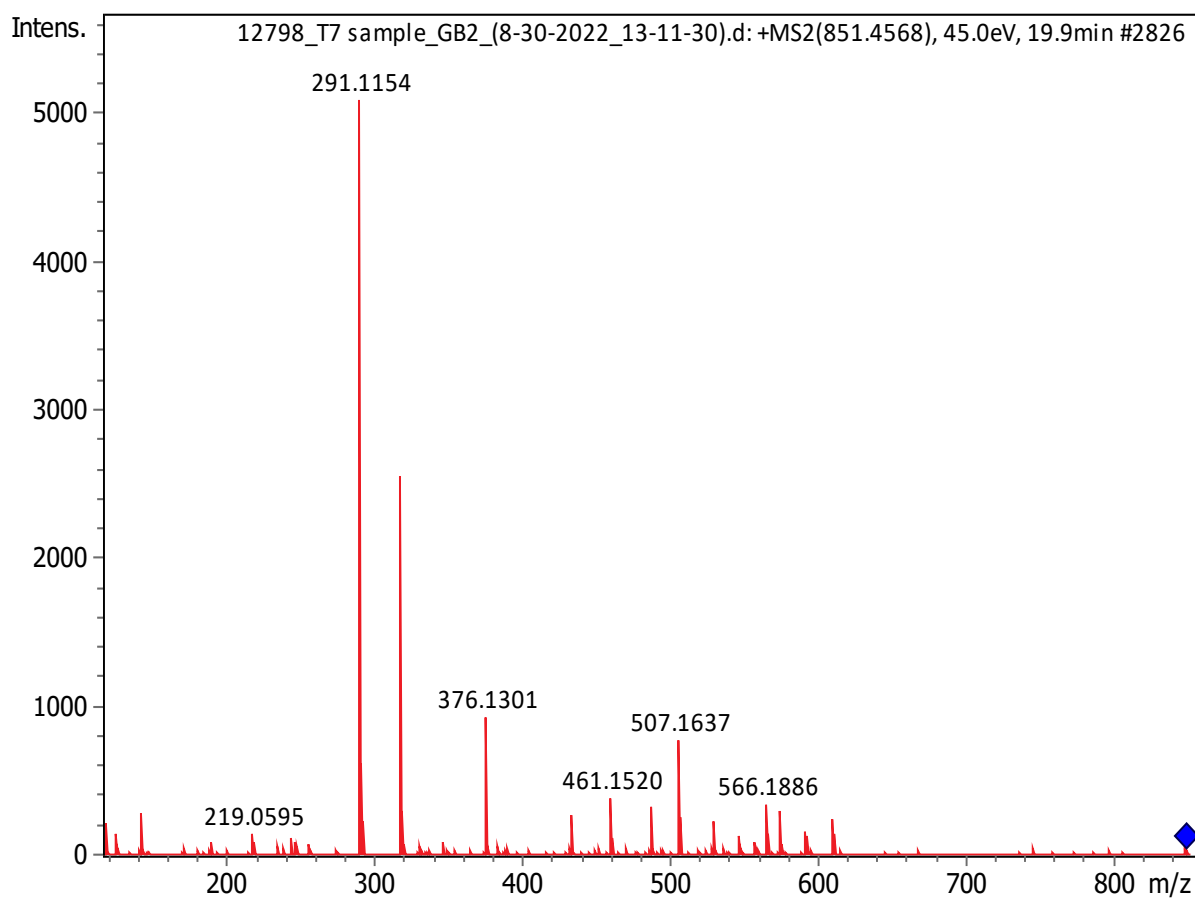


Fig.31.: Fragmentation spectrum of photolytic fragment 851 m/z obtained from the HPLC-MS/MS analysis of T_7 sample during the reaction kinetics experiment.

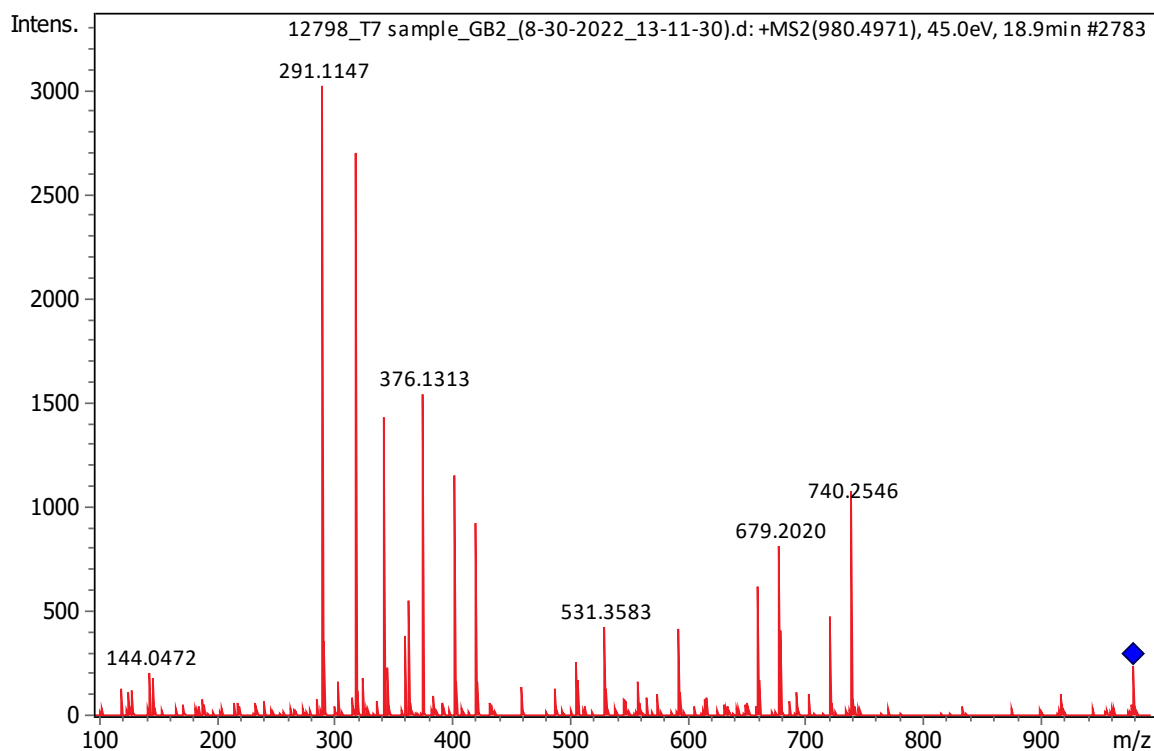


Fig.32.: Fragmentation spectrum of photolytic fragment 980 m/z obtained from the HPLC-MS/MS analysis of T₇ sample during the reaction kinetics experiment.

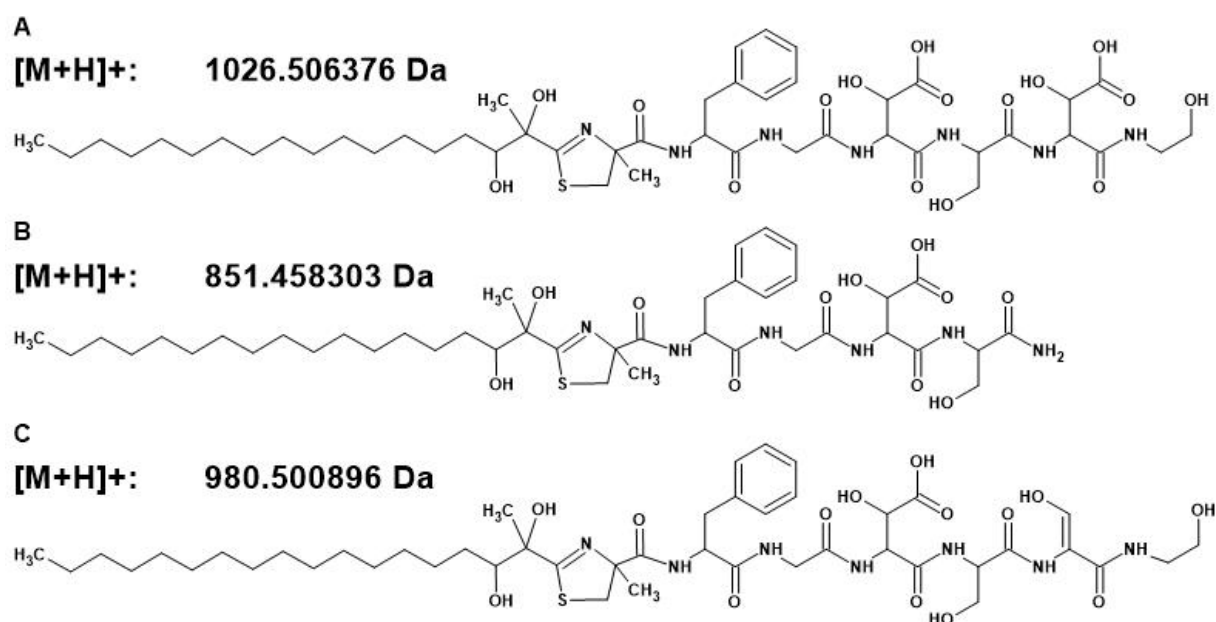


Fig.33.: A putative chemical structure of cyanochelin 1026 (A) and fragments 851 m/z (B) and 980 m/z (C)

The fragments were detected during the analysis of T₀ (633 m/z, 634 m/z) and T₁ (851 m/z, 980 m/z) samples and were subsequently detected in all following samples with

increasing signal intensity in time. Base on the measured peak area the molecules 633 m/z and 634 m/z represent the predominant photoproducts of the photolytic decomposition of cyanochelin 1026. The increasing signal measured for peaks of these two compounds can be seen in Fig.30 together with decreasing signal of cyanochelin 1026 molecule. The signal of photoproducts 851 m/z and 980 m/z was detected significantly lower than for previously mentioned photoproducts and there has been no tendency to increase in time observed.

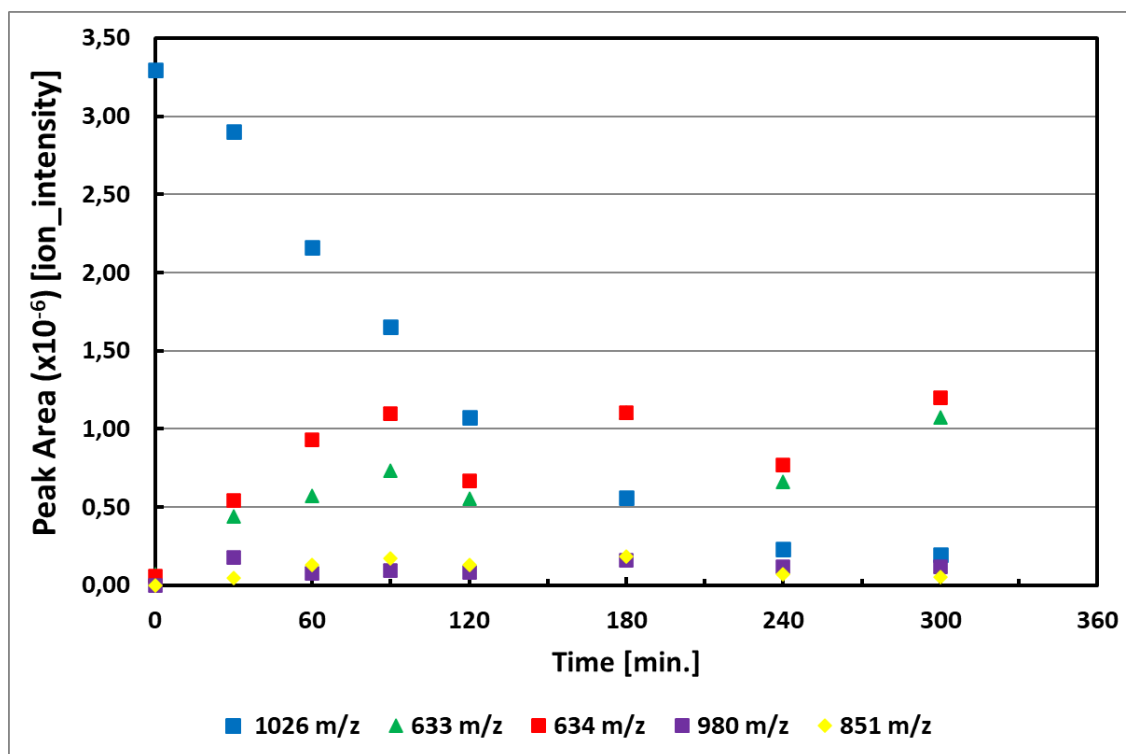


Fig.34.: The development of peak area of a signal measured for cyanochelin 1026 m/z, and its photolytic fragments in time during the photolytic experiment

5.4 Evaluation of stoichiometry of Fe^{3+} cations photoreduction facilitated by cyanochelin m/z 1026 and identification of photolytic activity of cyanochelin m/z 1026 photolytic fragments

The photodegradation of cyanochelin 1026, when exposed to the UV light in complex with Fe^{3+} results in formation of various photolytic fragments and reduced iron. The subsequent chelation and photolytic activity of resulting cleaved siderophore towards the Fe^{3+} cations is questionable (Hardy et al., 2019) (Galica et al., 2021). Such an activity would lead to non-equimolar ratio between amount of cyanochelin 1026 amended to the reaction solution and amount of Fe^{2+} cations produced. Thus, the

stoichiometry of Fe^{3+} photoreduction was assessed through measurement of Fe^{2+} concentration with a photometric method using complex of Fe^{2+} with bathophenanthroline disulfonic acid (BPDS).

5.4.1.Optimisation of Fe^{2+} detection with bathophenanthroline disulfonic acid (BPDS)

Within the optimisation of BPDS detection method the absorbance was tested for three forms of iron prepared in various concentrations to find the most fitting form for calibration standard. The absorbance of BPDS- Fe^{2+} complex was found to increase linearly with the concentration of Fe^{2+} in the solution up to $c(\text{Fe}^{2+})=100 \mu\text{M}$ (Fig.35). The measured absorbances were observed to differ among the respective compounds by approximately 9%, $\text{RSD}<9\%$ (Tab.S3), which can be considered as a negligible difference. The result from this experiment implies that all the studied forms of iron can be used as a calibration standard for stoichiometric experiments. At the end, the ferrous sulphate was chosen as standard compound, since it contains iron in divalent form, which can be directly complexed with BPDS with no reduction with hydroxyl amine required.

Second step of optimisation assessed the stability of BPDS- Fe^{2+} upon exposition to UV-Vis light conditions, which will be set during the stoichiometric experiment. The absorbance of BPDS- Fe^{2+} complex remained constant, $m=1,97$, during the whole experiment with variability less than 2 %, $\text{RSD}=1,93\%$ (Tab.S3) (Fig.36). In conclusion, the optimisation showed positive results, which permits to use the BPDS based colorimetric method for determination of Fe^{2+} in aqueous solutions in demanded concentration range.

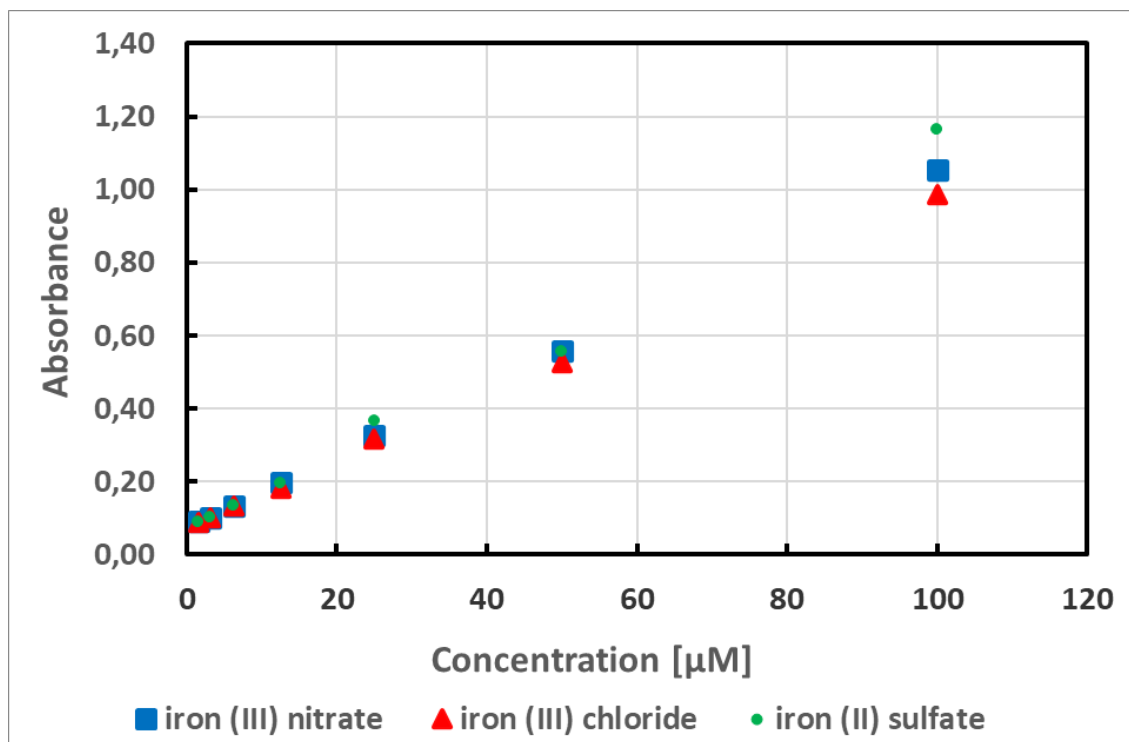


Fig.35.: Absorbance of BPDS-Fe^{2+} complex (535 nm) in concentration range 1,56-100 μM prepared from three various iron containing salts- iron (III) nitrate, iron (III)chloride and iron (II) sulfate

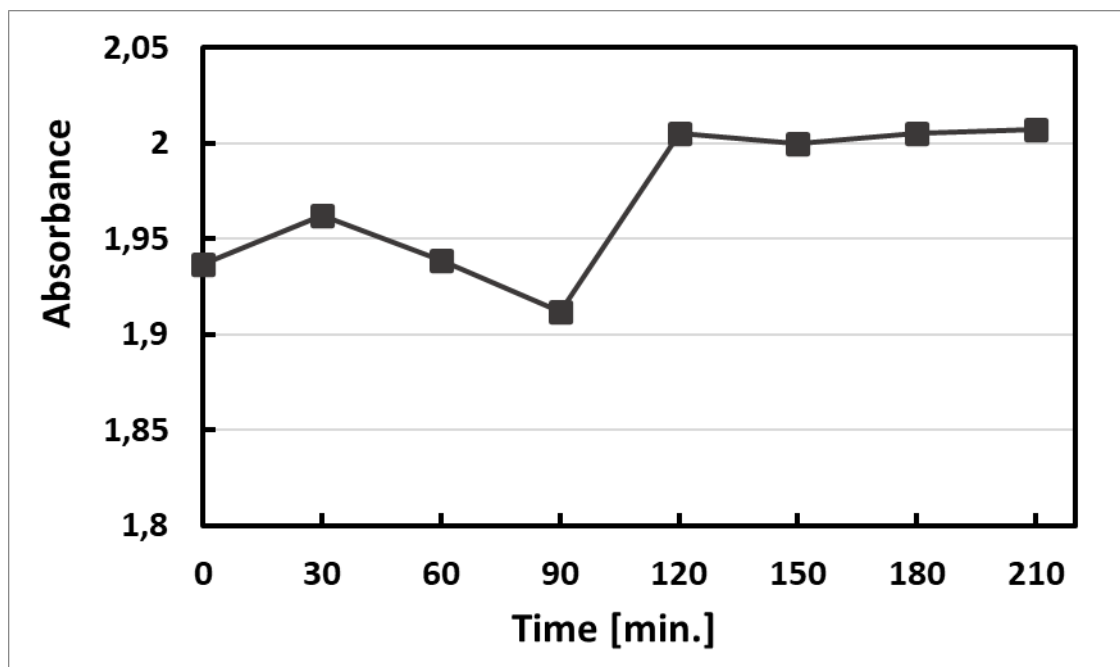


Fig.36.: The absorbance of BPDS-Fe^{2+} complex (535 nm) measured upon constant exposition to UV-Vis light for 210 minutes

5.4.2. Evaluation of stoichiometry of photoreduction of Fe³⁺ cations facilitated by cyanochelin m/z 1026

The stoichiometry of Fe³⁺ photoreduction was assessed in experiment, when 50 µM cyanochelin solution was amended with various concentrations of Fe³⁺ (25, 50, 75, 100, 125 and 150 µM) in three different reaction mixtures (BPDS+cyanochelin 1026, cyanochelin 1026 only and BPDS only). Subsequently all mixtures were exposed to UV light for four hours. Calibration curve was prepared in concentration range 12,5-150 µM from ferrous sulphate solution amended with BPDS by measurement of BPDS-Fe²⁺ absorbance (535 nm). The concentration of Fe²⁺ was calculated from the equation derived from calibration curve equation $y = 0,0131x + 0,0708$ (Fig.37).

$$y = 0,0131x + 0,0708$$

$$x = \frac{y - 0,0708}{0,0131}$$

x..... Fe²⁺ concentration [µM]

y.....peak area

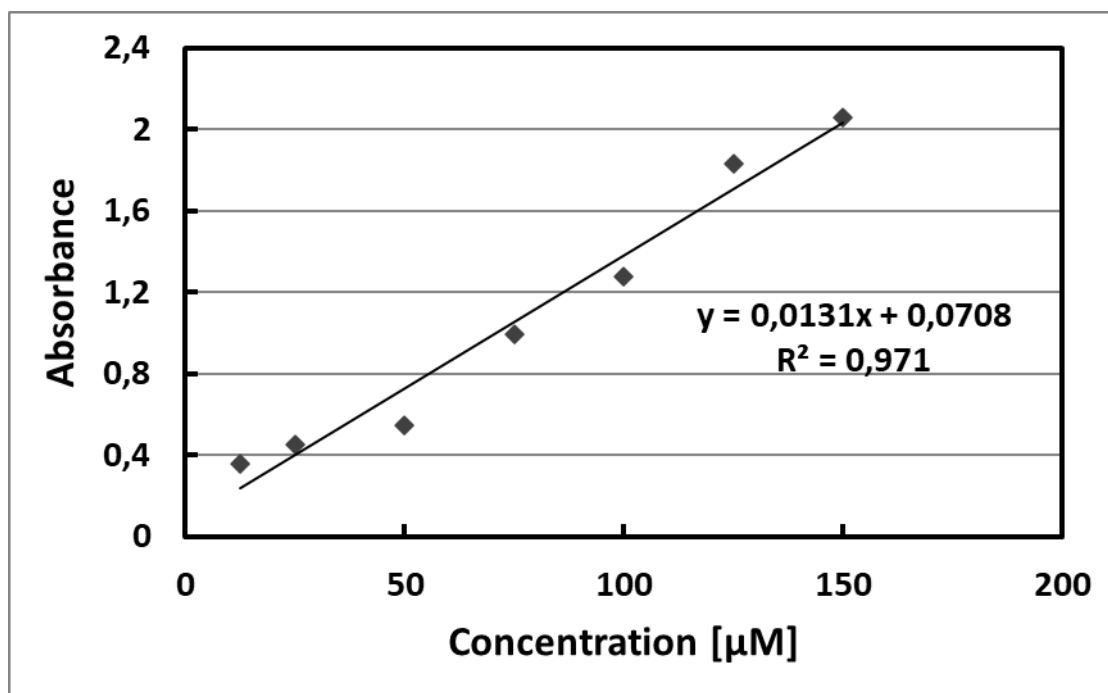


Fig.37.:The calibration curve for determination of Fe²⁺ concentration within the stoichiometric experiment

The first reaction mixture (cyanochelin+BPDS) has showed a gradual increase in amount of produced Fe^{2+} along with increasing concentration of Fe^{3+} in the reaction solution (Fig.38). The highest concentration was detected in the solution with initial concentration of Fe^{3+} set on $c(\text{Fe}^{3+})=150 \mu\text{M}$. Concentrations of formed Fe^{2+} in this reaction mixture represent 50-75% from the initial concentration of Fe^{3+} present in the reaction mixture (Tab.S4). In the case of a second reaction mixture (cyanochelin 1026 only), when the BPDS was amended after the UV-light exposition, the results showed almost no formation of Fe^{2+} cations during the UV light exposition (Fig.38). The Fe^{2+} cations were detected only in the case of samples with $c(\text{Fe}^{3+}) \geq 100 \mu\text{M}$, which corresponds to 2:1 molar ratio between Fe^{3+} and cyanochelin respectively. In the samples with $c(\text{Fe}^{3+}) < 100 \mu\text{M}$ the concentrations of Fe^{2+} were close to zero. The concentration of Fe^{2+} produced during the reaction has not exceeded 12% from the initial concentration of Fe^{3+} present in the reaction mixture (Tab.S4). Contradictory results were observed in negative control (BPDS only) (Fig.38). In the control reaction mixture, a formation of significant amount of Fe^{2+} was detected, which was observed to elevate along with the increasing concentration of Fe^{3+} in the reaction solution. The highest amount of Fe^{2+} was produced in the sample with $c(\text{Fe}^{3+})=150 \mu\text{M}$. The concentration of Fe^{2+} , which was produced during the photoreduction, fluctuates between 28-42% from the initial concentration of Fe^{3+} present in the reaction mixture (Tab.S4).

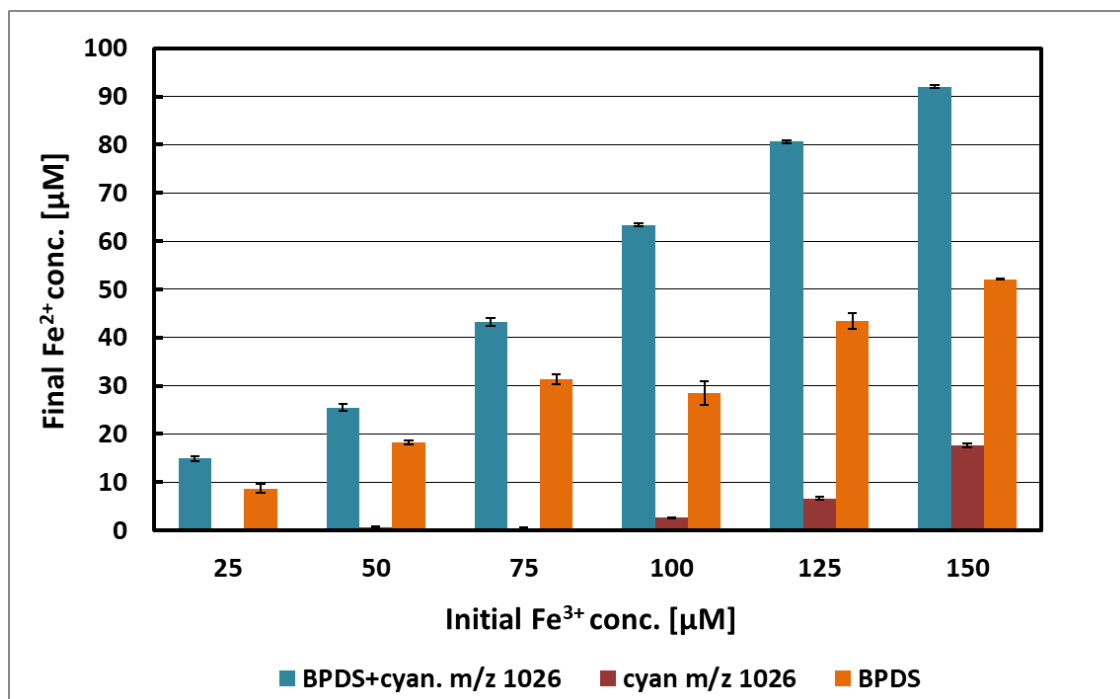


Fig.38.: The relation between amount of Fe^{3+} cations amended to the reaction solution and amount of Fe^{2+} formed in time upon exposition of samples to UV-Vis light. Abbreviations: conc.-concentration, BPDS- bathophenanthroline disulfonic acid, cyan.1026- cyanochelin 1026

The stoichiometric experiment has shown unexpected behaviour of BPDS and Fe^{2+} in reaction solution. According to the results of control reaction mixture shown on Fig.38 the BPDS can facilitate the reduction of the Fe^{3+} on its own. The results from second reaction mixture (cyanochelin only) showed very low concentrations of detected Fe^{2+} , which conflicts the results from kinetics experiments (chapter 5.2.2) supposing the production of Fe^{2+} to be equimolar to cyanochelin 1026 concentration. Thus, a precipitation of Fe^{2+} after the photoreduction has probably occurred due to inappropriate conditions in the aqueous solution, which became a point of subsequent analysis.

5.4.3. Analysis of Fe^{2+} detection problems from stoichiometric experiment

The result of the previous experiments suggested hypothetical problems with Fe^{2+} detection in the stoichiometric experiments. Two reasons of this complications have been considered, the back oxidation from Fe^{2+} to Fe^{3+} and the second the influence of the buffer solution. The first experiment considers the back-oxidation of Fe^{2+} to Fe^{3+} in oxygenic atmosphere. The absorbance of nitrogen treated sample was detected at value near 0,06 ($m=0,057$, $n=3$, $SD=0,002$) (Fig.39). This value is according to the calibration

curve prepared for the previous experiment (Fig.37) implies that the concentration of Fe^{2+} in nitrogen treated solutions is approaching zero value. A very similar results were observed in the case of untreated samples. In this case, the absorbance was measured in vicinity of 0,05; ($m=0,049$, $n=3$, $SD=0,004$) (Fig.39), which is only 0,08 from the value measured for nitrogen treated samples. This fact induces that the concentration of Fe^{2+} in the solution is reaching the zero value.

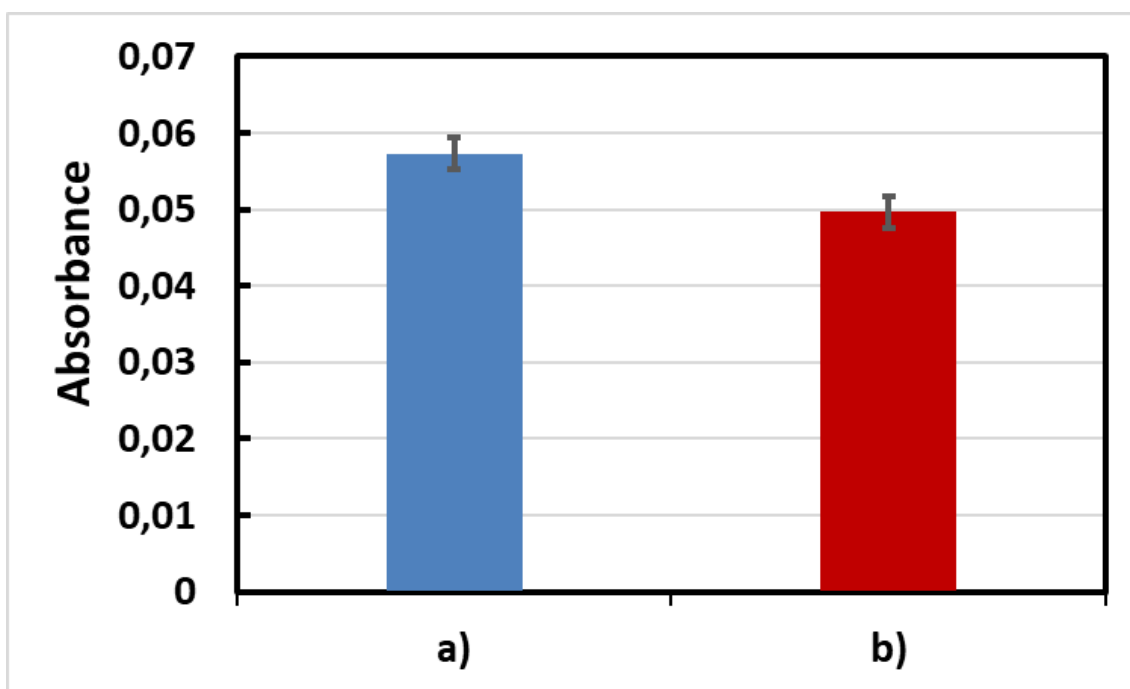


Fig.39.: The absorbance of BPDS- Fe^{2+} complex (535 nm) measured after addition of BPDS to a) Fe^{2+} MOPS solution treated with nitrogen, b) Fe^{2+} MOPS solution without nitrogen treatment

The second experiment was designed to prove the influence of buffer on the stability of Fe^{2+} cations in the aqueous solution. The absorbance for MOPS amended samples was measured around 0,05 ($m=0,045$, $n=3$, $SD=0,02$). This value is again below the detection limits of BPDS detection method and thus the concentration of Fe^{2+} is close to zero. A different situation occurred in the case of samples prepared in dH_2O without MOPS amendment. In this case the absorbance in samples was detected near 0,6 ($m=0,59$, $n=3$, $SD=0,016$) (Fig.40). This value was approximated using the calibration cure (Fig.37) to correspond to approximately 50 μM , which represents one half of the initial concentration (100 μM).

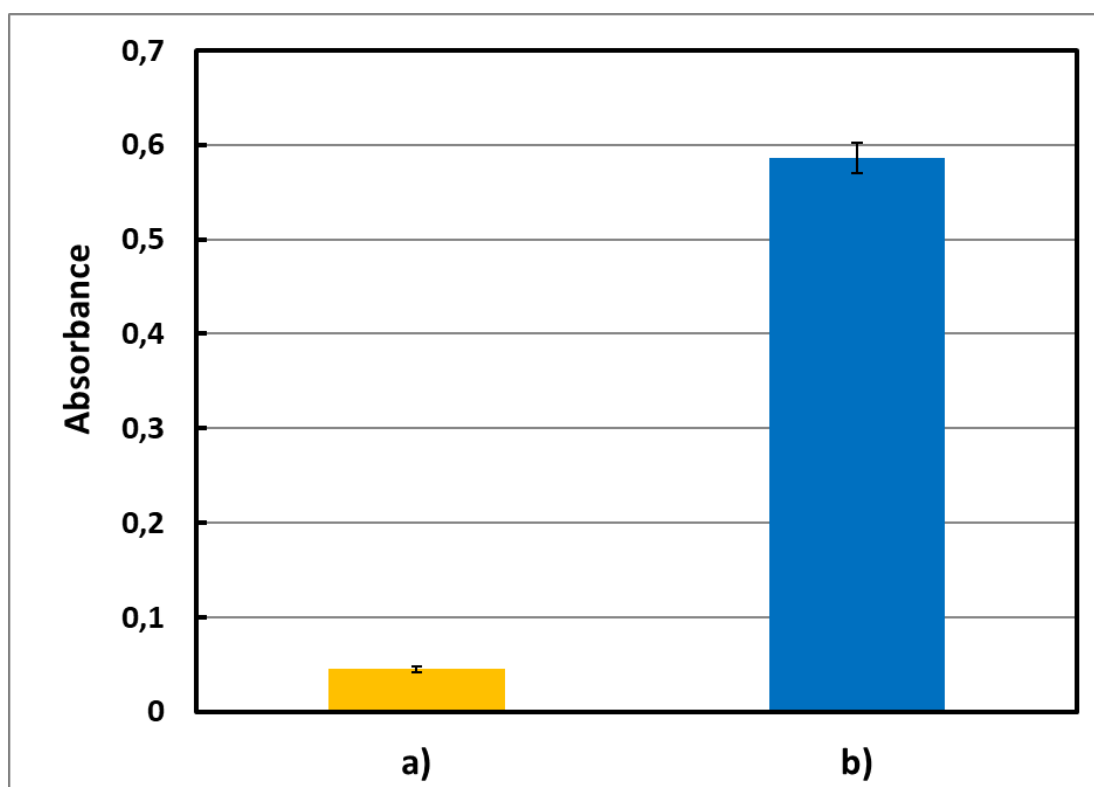


Fig.40.: The absorbance of BPDS-Fe²⁺ complex (535 nm) measured after addition of BPDS to Fe²⁺ solutions prepared in a) MOPS buffer, b) dH₂O

6. Discussion

6.1 Optimisation of cyanochelin 1026 extraction procedure and isolation yields

This thesis was focused on photoreaction of cyanochelin 1026-Fe³⁺ complex upon exposition to the UV light. Since considerable amount of pure compound was required for the subsequent kinetics and stoichiometric experiments and analysis of the compound structure with NMR, the optimisation of the isolation procedure was stated as a first aim of the thesis. The first step of the isolation procedure, which was optimized, was the determination of the solvent with the highest extraction capacity, which was then utilized for extraction of cyanochelin 1026 from dried *Leptolyngbya* biomass. According to the literature two organic solvents utilized in extraction of lipopeptide siderophores-acetonitrile (Galica et al., 2021) and methanol (Kreutzer et al., 2012), were chosen to be tested in various ratios with water for the extraction capacities. The results from the HPLC-MS/MS data (Fig.14 and Fig.15) showed that the 60% acetonitrile represents the most suitable solvent for the cyanochelin 1026 biomass extraction and was subsequently utilized for cyanochelin extraction. The utilization of the acetonitrile as an extraction solvent is not a common approach for extraction of lipopeptides, since many extraction of these amphiphilic molecules were performed using MeOH as in the case of Cupriachelin (Kreutzer et al., 2012), aquachelins (Martinez et al., 2000), synechobactins (Ito et al., 2005), etc.

Within the second step of the extraction the liquid- liquid phase partitioning was performed at two different pH conditions. At first the basic phase extraction was performed at pH=10. The reason, why such a pH was applied, is a behaviour of β -hydroxy aspartic acids with respect to the pH of the solution. Upon basic pH the carboxyl containing amino acids prevail in ionic form, since the pKa value of such amino acids is usually found in vicinity to pKa=5 (Miethke et al., 2007). This approach is commonly used in industrial separation of organic compounds with different pKa values. The polar form of cyanochelin 1026 migrates during the phase extraction to the water phase, where it is separated from the contaminants such as chlorophylls, carotenoids etc, which go to the organic phase. In the following two extraction steps the pH value was decreased to pH=3, which caused depolarization of cyanochelin molecule based on the principle described above, and thus the cyanochelin 1026 moved to the

organic phase. The organic solvent could be subsequently removed by rotary evaporation resulting in extract volume reduction. The pure compound was subsequently isolated from the extract using HPLC in two steps.

The isolation procedures yielded 80 mg of cyanochelin 1026. The isolation procedures, which were carried out percussively during the work on the thesis, utilized approximately same amount of biomass for each batch with, which has resulted in variable yields of cyanochelin 1026 as can be seen in Tab. 11. The purity of the isolated fractions was tested with HPLC-MS/MS analysis. One of the isolated fractions was sent to Dr. Valeria Costantino PhD. from “TheBlueChemistryLab”, Department of Pharmacy, University of Naples Federico II, Napoli, Italy, where the 2D structure of the cyanochelin 1026 lipid moiety was revealed using NMR method. The NMR analysis has identified two hydroxy groups present on the β and γ positions of the lipidic moiety. The hydroxylation of lipid moiety is a common feature of cyanobacterial lipopeptides originating from PKS, which can be found in various groups of cyanobacterial lipopeptides such as Puwainaphycins (Hrouzek et al., 2012), Hassallidins (Neuhof et al., 2005), etc. These hydroxyl groups might form the two so far not known coordination bonds between cyanochelin 1026 and Fe^{3+} (Fig.16), however, the stereochemistry of the molecule would have to be known to verify this statement, which is still point of further investigation.

6.2 Determination of reaction kinetics of cyanochelin m/z 1026- Fe^{3+} complex decomposition upon exposition of UV-light

The kinetics of the photodegradation of cyanochelin 1026 to photoproducts was described within two experiments. Within the first experiment, which was considered as an optimisation experiment, the samples were analysed with HPLC-DAD method without any post reaction treatment. This first problem, we have tackled within the pivotal experiment, was a poor detection of the cyanochelin 1026 on the DAD detector in all samples including the calibration samples prepared in MeOH. The reason for the poor detection was a C-18 stationary phase, which was used within the pivotal analyses. The C-18 column was found to have strong retention for noncomplexed cyanochelin 1026, which caused stacking of the compound in the column. The explanation for such a strong retention might be an acidic pH used during HPLC analysis in combination with unpolar C-18 stationary phase. The low pH caused neutralization of cyanochelin 1026

molecule to unpolar state, which reduced the affinity to the mobile phase, while the C-18 moieties facilitated a fixation of the cyanochelin 1026 on the column particles. The utilization of C-18 column would not be problem in neutral pH range, since it was reported to be used within HPLC analysis of fatty with satisfactory results (Takashima et al., 2018). The problem was fixed with the Pentafluorophenyl (PFP) stationary phase, which attracts the cyanochelin 1026 molecule through aromatic ring interactions between phenylalanine and pentafluoro phenyl group and is not affected by the pH of the mobile phase (Euerby et al., 2007).

Despite the PFP column has improved the detection of cyanochelin 1026 during the HPLC analysis, the problems with detection were observed during the first photolytic experiment again, however, this time the problems were limited only to experimental samples but not to the calibration standards. During the analysis UV-Vis samples the concentration of cyanochelin 1026 has decreased within the analysis of the samples from UV-Vis reaction mixture to one half of the initial concentration and was stable trough the experiment in Vis and dark reaction mixtures (Fig.18), moreover, surprising trends (initial increase and sudden drop-down) was observer in UV-Vis conditions (chapter 5.2.1).The precipitation of cyanochelin 1026 in aqueous solution was considered as the most probable reason for loss of the cyanochelin 1026 molecules from samples during the analysis. The cyanochelin 1026 as a lipopeptide belongs to natural biosurfactants due to the amphiphilic properties of the molecules. These compounds can form micelles in aqueous solutions or attach to the surface of the wall of the MS vial, which can cause vanishing of lipopeptide from the solvent (Zhu et al., 2021). The precipitation of cyanochelin 1026 might be an explanation for loss of the compound from analysed samples. As a solution of this problem, a post reaction treatment of samples with dilution with MeOH in 1:1 volumetric ratio, pH adjustment using NaOH and treatment with ultrasonication bath was suggested, since both applied changes increase the solubility of cyanochelin 1026 in aqueous solution as describe before.

The photoreaction in the second experiment was performed with the same approach as in the first photoreaction, however, the post reaction treatment and HPLC-MS/MS analysis was employed to achieve reliable detection. The results of this experiment (Fig.23) gave us information about the decomposition of cyanochelin 1026-Fe³⁺ complex and photolysis of cyanochelin molecule in time upon exposition to UV light. The initial concentration measured at T₀ is adequate to the value, which was established

before the reaction was initiated (50 μ M), which confirms a good detection of HPLC-MS/MS analytical method in combination of post reactional treatment of samples. The concentration of cyanochelin 1026 in time was observed to be declining in time up to T₆, when the concentration reached zero value. The concentration seems to decline linearly between T₁ and T₅, which indicates that the photoreaction proceeds in zero order, and it is independent of concentration of cyanochelin 1026. This statement was lately confirmed when the rate of reaction was determined using integration method. Our results show that the photodegradation of the cyanochelin 1026-Fe³⁺ complex is a reaction of zero order with reaction rate constant $k=4,89 \times 10^{-3} \text{ M.s}^{-1}$, which means it is independent on concentration of cyanochelin 1026. The reliability of this result might be contested by a low reliability value determined in the case of Fig.24 ($R^2= 0,014$). The experiment reliability could be improved by analysis of samples in triplicates, however, this solution is not feasible, since it would prolong the analysis time to approximately 30 hours, which might cause troubles in lately analysed samples as they are rather unstable at room temperature. The information about the reaction kinetics of cyanochelin 1026 photodegradation might be very useful for the explanation of the exact role of this compound in the environment, which was not clarified till now.

As the conditions (mostly the UV light intensity) used in the experiment were selected to approximate values which might occur in nature (an average bright sunny day, UV360 nm = 15 W.m²) it can be concluded that the half-live of the molecule in water solution will be approximately 89 minutes. This suggest that under photic conditions cyanochelin and similar molecules will be readily photolytically cleaved and might not assure specific delivery back to the producer cells unless they are protected. In this context it might be important to note that many cyanobacterial cells possess large mucilaginous sheets containing sunscreen pigments which might protect and harbour such siderophores.

6.3 Identification of photolytic fragments of cyanochelin m/z 1026-Fe³⁺ complex degradation with HPLC-MS/MS

As mentioned in the theory section (chapter 2.3.5), the photoreduction of Fe³⁺ accompanied by siderophore degradation results in formation of photolytic products, which can still maintain the affinity towards Fe³⁺ cations as described in case of citrate containing siderophores such as ochrobactins (J. D. Martin et al., 2006), synechobactins

(Ito et al., 2005) or aerobactin (Butler et al., 2021). Within the second experiment focused on reaction kinetics the HPLC-MS/MS analytical method was employed. The undeniable advantage of MS/MS detection is the ability to monitor on the detector various molecules in predefined mass range, which can be subsequently identified via their fragmentation spectra.

Four photolytic fragments of cyanochelin 1026 were identified based on the comparison of cyanochelin 1026 fragmentation spectra (Fig.27). The structure of fragments 633 m/z and 634 m/z was revealed in previous study focused on cyanochelin 1026 and its photoreactivity (Galica et al., 2021). Since the analysis was carried out in positive mode, the measured m/z values correspond to molecular weights 632 Da and 633 Da. This fact indicates, that the cyanochelin molecule (1025 Da) was cleaved at peptide bond between glycine and β -hydroxy aspartic acid and thus the carboxylate moiety donated by the β -hydroxy aspartic acid is further not valuable in the resulting molecule. This would cause losing the ability to coordinate the Fe^{3+} cation, however, this statement is questionable, since more ligands can be involved in iron (III) chelation (Carrano et al., 1978). The structure of fragments 851 m/z and 980 m/z was revealed based on the identification of structure of signal ions according to the structure of fragments of cyanochelin 1026 described in Fig.28. The structure of fragment 851 m/z presented at Fig.33 suggests that the cleavage has occurred between β -hydroxy aspartic acid and serine. This finding is supported by the literature data, which reports the photolytic cleavage of β -hydroxy aspartate containing siderophores to be localized in the vicinity of β -hydroxy aspartic acid as described in the case of aquachelins (K. Barbeau et al., 2001). The cleavage of the β -hydroxy aspartic acid has been also reported to be associated with oxidation of β -hydroxy aspartic acid (K. Barbeau et al., 2001), however, this cannot be confirmed since the second product of this cleavage was not identified. The structure of 980 m/z presented in Fig.33 suggests a different reaction machinery than described in the case of fragment 851 m/z. The cleavage of cyanochelin 1026 has occurred on the same amino acid as in the previous case, but this time the oxidation of β -hydroxy aspartic acid has occurred without degradation of the peptide bond between β -hydroxy aspartic acid and serine and cleavage of the cyanochelin molecule into two parts. The loss of the CO_2 was confirmed in the case of citrate containing siderophores such as synechobactins (Ito et al., 2005) or aerobactin (Butler et al., 2021), however, there is no evidence for this phenomenon to not be observed in the

case of β -hydroxy aspartic acid containing siderophore. Moreover, the oxidation of β -hydroxy aspartic acid was reported to occur during photodegradation of aquachelins (K. Barbeau et al., 2001).

Fragments 851 m/z and 980 m/z represents a putative iron(III) chelators, since the major part of the cyanochelin 1026 molecule was kept intact and some of the remaining hydroxyl group might be potentially in appropriate position to substitute the loss of the of β -hydroxy aspartic acid carboxylate moiety. This indicates the coordination of Fe^{3+} could be still feasible. The chelation activity of both is, however, questionable, since in both cases one β -hydroxy aspartic acid is either missing completely (851 m/z) or structurally degraded by oxidation (980 m/z). As described in the literature the coordination of central atom proceed through specific moieties on the siderophore molecule, which are not randomly localized on the molecule, but they occupy an exact parts of the compound (Sandy et al., 2009). This implies that the bare presence of hydroxyl groups on the molecule of the photolytic fragments does not promise the coordinating abilities of the fragment.

From the quantitative point of view the fragments 633 m/z and 634 m/z represent the major products of photolytic decomposition of cyanochelin 1026. The increase of signal of these fragments in time is shown on Fig. 34 along with the decrease of cyanochelin 1026 signal. The signal for both mentioned fragments is increasing from T_0 to T_3 , where it stops. For the rest of the analysis the signal was observed to be fluctuating around the value 1×10^6 peak area (634 m/z) and $0,75 \times 10^6$ peak area (633 m/z) and without any subsequent increase. The reason for this observation is not clear so far and requires further analysis with more focus on these fragments. Despite the fragment may not be able to coordinate the iron (III) atom on its own, there is still a possibility of formation of non-equimolar complexes when the electron donating groups are provided through more ligands at once (Carrano et al., 1978). Thus, there is still chance for the listed fragments to be involved in iron (III) chelation. The fragments reported above may not be the only fragments, which are formed during the photodecomposition of cyanochelin 1026 molecule, however, the MS data collected from kinetics experiment did not show them. A suggestion for further investigation would be to elevate the amount of cyanochelin 1026 used in the experiment, which would increase the concentration of photolytic fragments in samples and thus increase their detection, however, this step would require further optimisations and isolation of sufficient material.

6.4 Evaluation of stoichiometry of Fe^{3+} cations photoreduction facilitated by cyanochelin m/z 1026

The evaluation of stoichiometry of the cyanochelin 1026 facilitated photoreduction of Fe^{3+} cations is the first step to elucidate the photoreductive potential of the formed fragments. The MS/MS analysis of samples from photoreaction experiment revealed four fragments (633 m/z, 634 m/z, 851 m/z and 980 m/z), which might be also involved in iron (III) photoreduction. Within the stoichiometric experiments the reactions were evaluated through detection of iron (II), which represents the common product for both cyanochelin 1026 or fragment facilitated photoreduction. The detection of iron (II) was carried out using bathophenanthroline disulfonic acid (BPDS) indicator. The BPDS detection method was classified to be suitable for the stoichiometric experiments based on the results from two preliminary assays. In addition this method was mentioned in several research papers as method used for determination of photolytic activity of siderophores domains (Kreutzer et al., 2012), (Hardy et al., 2019) or (Galica et al., 2021).

The first assay was focused on the determination of concentration range with linear correlation between the concentration and absorbance of BPDS- Fe^{2+} complex using three various sources of Fe^{2+} . The absorbance of BPDS- Fe^{2+} complex was found to be linear up to concentration 100 μM as it can be seen of Fig.35. Such a concentration range is sufficient for the following stoichiometric experiment, since the cyanochelin 1026 concentration in reaction solutions was 50 μM . Based on this finding the BPDS detection method was considered suitable for the stoichiometric experiment. Within a second assay stability of BPDS- Fe^{2+} complex was tested under the exposition to UV light. The results, which can be seen in Fig.36, confirmed that the BPDS- Fe^{2+} complex does not undergo any degradation upon exposition to UV light, which would cause significant problems during the Fe^{2+} detection.

The stoichiometric experiment was performed in triplicates with three reaction mixtures with various initial composition of reagents and BPDS indicator. The absorbance measured for samples collected from respective reaction solutions were used to determine the total Fe^{2+} produced within the photoreaction. The determined concentrations of Fe^{2+} in reaction solutions with various initial Fe^{3+} concentrations are shown in Fig.38. The highest concentrations of Fe^{2+} were detected in the first reaction

mixture. In this case the amount of Fe^{2+} was linearly increasing with the amount of Fe^{3+} amended into the reaction solution until the solution with the highest Fe^{3+} : cyanochelin ratio (4:1). The average increment of Fe^{2+} within this reaction mixture indicates that the reaction did not proceed in equimolar ratio, since the concentration of produced Fe^{2+} exceed the concentration of cyanochelin 1026 in reaction solution ($50\mu\text{M}$, see Fig.38). This finding would confirm the photoreactivity of cyanochelin photolytic fragments towards the Fe^{3+} cations, however, this consideration was subsequently rejected based on the results obtained from the third reaction mixture, which was supposed to represent a negative control. Despite the cyanochelin 1026 was excluded from the reaction solutions of third reaction mixtures, the results showed elevated concentrations of Fe^{2+} in reaction solutions after exposition to the UV light (Fig.38). The concentration of Fe^{2+} in the analysed samples was observed to be increasing with the initial concentration of Fe^{3+} and reached the maximum also at the highest Fe^{3+} : cyanochelin ratio (4:1). This finding suggests that the photoreduction of Fe^{3+} in first reaction mixture was performed rather via BPDS than cyanochelin 1026. The troubles spurred more detailed literature search to see if this was observed before. The literature search reports the unspecific reduction of Fe^{3+} cations by BPDS indicator and recommended to avoid this compound in long time lasting experiments (Coward et al., 1993). This problem was supposed to not occur in second reaction mixture, where the BPDS was amended after the UV light exposition, however, the results showed surprisingly low amount of produced Fe^{2+} . In the case of second reaction mixture the Fe^{2+} was detected just in the samples with Fe^{3+} : cyanochelin ratio $\geq 2:1$. This result conflicts with data with kinetics experiment, where we have observed almost 100% consumption of cyanochelin 1026 after three hours of exposition to UV light (Fig.23), which is supposed to produce the equimolar amount of Fe^{2+} . Based on this assumption the loss of produced Fe^{2+} during the reaction was considered as the reason for such a low concentration in samples of second reaction mixture.

The reasons for such a significant loss of the Fe^{2+} were subsequently investigated experimentally based on the theoretical assumptions. According to the theory Fe^{2+} cations is very vulnerable to oxidation back to Fe^{3+} form, when the cation is found in oxygenic atmosphere, which significantly shortens the lifetime of Fe^{2+} in aqueous solution (B. Morgan et al., 2007) and might be a reason for vanishing of Fe^{2+} from the samples. Based on this information the detection of Fe^{2+} was compared between MOPS

solution treated with N₂ flux and untreated MOPS solution. The results described in Fig.39 showed no influence of N₂ treatment on the detection of Fe²⁺, since the absorbances measured in both reaction mixtures reached the vicinity of detection threshold. The results from this experiment rejected the assumption about loss of the Fe²⁺ due to the back oxidation to Fe³⁺ form. As another reason for vanishing of the Fe²⁺ from the solution, the presence of MOPS buffer was suggested. This suggestion was based on the fact, that the MOPS buffer works as an additional source of hydroxyl anions in aqueous solutions, which can form water insoluble complexes with Fe²⁺ at neutral pH (B. Morgan et al., 2007). Based on this assumption the detection was compared between MOPS solution and water solutions containing the same amount of Fe²⁺ cations. The results of this experiment are shown in Fig.40. The measured absorbance was found significantly different between both reaction mixtures reaching the value of 0,6 in the case of Fe²⁺ water solution, while the absorbance in MOPS solution was measured in vicinity to threshold of the detection method. This finding confirms the previously stated assumption about complexation of Fe²⁺ to water insoluble complexes, which cause its vanishing from the reaction solution. Despite the absorbance measured for water solution reached significantly higher values than in the case of MOPS solution there is still a considerable amount of amount of Fe²⁺ missing, since the measured absorbance corresponds to approximately to 50 μM (according to the calibration curve on Fig.37), which is one half of the initial concentration (100 μM). Thus, the water solution still does not provide sufficient solvent for the stoichiometric experiment and further investigations must be carried out to describe the production of Fe²⁺ within the cyanochelin 1026 mediated photoreactions quantitatively.

The unspecific BPDS facilitated photoreduction of Fe³⁺ might seem to contest the evaluation of photoreactivity of siderophores from previously mentioned research papers, however, in the case of these papers the BPDS was amended to the reaction mixtures after the reaction was carried out. These reactions were performed in non-equimolar reaction mixtures, where siderophore concentration was established to be in excess to Fe³⁺ concentration. The BPDS was used only as an indicator of Fe²⁺ presence in the reaction mixture and no quantification based on the BPDS-Fe²⁺ complex absorbance was performed. This implies that only a part of the formed Fe²⁺ was detected, which was considered as a positive result.

7. Conclusion

This master thesis was focused on description of kinetics of photodegradation of cyanochelin 1026-Fe³⁺ complex, identification of cyanochelin 1026 photoproducts and assessment of their photoreductive potential towards Fe³⁺ cations via stoichiometric experiments. Within a first part of the thesis 80 mg of cyanochelin 1026 were isolated in sufficient purity after the optimisation of extraction procedure, which were subsequently utilized for kinetics and stoichiometric experiment and structure elucidation with NMR method. According to the kinetics experiment the photoreaction of cyanochelin 1026 was revealed to be zero order reaction with reaction rate constant $k=4,89 \times 10^{-3} \text{ M.s}^{-1}$ and half time of the photoreaction $t_{1/2}=5,32 \times 10^3 \text{ s}$. Within the subsequent analysis of MS data from kinetics experiment four photolytic fragment with photoreductive potential were identified. The photoreductive potential of cyanochelin 1026 photolytic fragments was not confirmed with the stoichiometric experiments due to the significant problems with Fe²⁺ detection in aqueous solution.

8. References

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Supplementary data

Tab.S1.: Peak areas measured from HPLC chromatograms (254 nm) within the optimisation of 1026 detection

UV-Vis		Vis		Dark	
Sample	Peak area	Sample	Peak area	Sample	Peak area
T0	345,9	T0	238,8	T0	237,6
T1	508,1	T1	367,6	T1	353,3
T2	583,1	T2	350,6	T2	360,7
T3	693,8	T3	325,1	T3	300,6
T4	482,9	T4	387,1	T4	351
T5	69,4	T5	342,8	T5	372
T6	38,7	T6	332,4	T6	335,1
T7	37,4	T7	312,6	T7	299,2

Tab.S2.: Concentration of cyanochelin 1026 in the second kinetics experiment derived from peak areas for signal of ion $[M+H^+]$ =1026 with calibration curve

Time of exposition [s]	Concentration [μ M]	Reaction rate constant		
		Zero order ($\times 10^{-3}$)	First order ($\times 10^{-3}$)	Second order ($\times 10^{-8}$)
0	52,41	NA	NA	NA
1800	45,56	3,81	4,67	1,59
3600	32,60	5,50	7,91	3,22
5400	23,71	5,32	8,81	4,28
7200	13,60	5,39	1,12	7,56
10800	4,569	4,43	1,36	18,53
14400	NA	NA	NA	NA
18000	NA	NA	NA	NA

Tab.S3.: Spectrometrically measured absorbances of BPDS-Fe²⁺ complex ($\lambda=535$ nm) formed from different iron compounds. Abbreviations: mean- mean value, SD-standard deviation, RSD-relative standard deviation

	Absorbance					
Concentration [μM]	Iron (III) nitrate	Iron (III) chloride	Iron (II) sulfate	Mean	SD	RSD
100	1,054	0,985	1,166	1,068	0,091	8,550
50	0,557	0,525	0,556	0,546	0,018	3,332
25	0,325	0,317	0,366	0,336	0,026	7,823
12,5	0,197	0,181	0,196	0,191	0,009	4,684
6,25	0,133	0,133	0,135	0,134	0,001	0,864
3,125	0,102	0,1	0,104	0,102	0,002	1,961
1,5625	0,091	0,089	0,09	0,090	0,001	1,111
6,25	0,133	0,133	0,135	0,134	0,001	0,864

Tab.S4.: The concentration of Fe^{3+} in reaction mixtures prepared at the beginning of the experiment and determined spectrometrically after the photoreaction and the consumption of the Fe^{3+} during the reaction expressed as percentual difference between final and initial value. Abbreviations: SD-standard deviation

BPDS+cyanochelin m/z 1026				
Concentration cyanochelin 1026 [μM]	Initial concentration of Fe^{3+} [μM]	Final concentration of Fe^{2+} [μM]	SD	Fe^{2+} increment [%]
50	25	14,901	0,536	59,603
50	50	25,486	0,764	50,972
50	75	43,247	0,780	57,662
50	100	63,349	0,344	63,349
50	125	80,651	0,289	64,521
50	150	92,025	0,289	61,350
50	200	152,662	1,042	76,331
Cyanochelin m/z 1026				
Concentration cyanochelin 1026 [μM]	Concentration Fe^{3+} [μM]	Concentration Fe^{2+} [μM]	SD	Fe^{2+} increment [%]
50	25	0	0	0
50	50	0,626	0,275	1,252
50	75	0,422	0,117	0,563
50	100	2,611	0,153	2,611
50	125	6,606	0,318	5,284
50	150	17,649	0,477	11,766
50	200	13,908	0,132	6,954
BPDS				
Concentration cyanochelin 1026 [μM]	Concentration Fe^{3+} [μM]	Concentration Fe^{2+} [μM]	SD	Fe^{2+} increment [%]
50	25	8,667	0,885	34,667
50	50	18,260	0,350	36,519

50	75	31,288	0,996	41,717
50	100	28,514	2,393	28,514
50	125	43,425	1,663	34,740
50	150	52,102	0,117	34,735
50	200	68,005	0,895	34,003