Chapter 2
Secondary Metabolites of Cyanobacteria and Drug Development

2.1 Secondary Metabolic Pathways Found in Cyanobacteria

Natural products, often called secondary metabolites, are low molecular weight organic molecules that have diverse and often very potent biological activities. Secondary metabolites are not essential for normal growth, development, or reproduction of an organism. They empower the producing organism to survive interspecies competition, provide defensive mechanisms against stress, and facilitate reproductive processes. Many secondary metabolites have proved invaluable as antibacterial or antifungal agents, anticancer drugs, cholesterol-lowering agents, immunosuppressants, antiparasitic agents, herbicides, diagnostics, and tools for research. Some of these have found to play a pivotal role in the treatment or prevention of a multitude of biological disorders. Many of the deadly diseases did not have any cure until these products were discovered. Secondary metabolites are commonly divided into structural classes related to their biosynthesis. This classification has its limitations because some compounds have building blocks from more than one biosynthetic pathway and some compounds that appear closely related can have completely different biosynthetic origins. The important classes of secondary metabolites are the polyketides and nonribosomal peptides, and other structural classes are alkaloids, terpenoids, shikimate-derived molecules, and amino glycosides. Recently, a new term "Parvome" has been proposed for these small molecules of great structural diversity (Davis and Ryan 2012).

Since the onset of the post-genomic era genomes of numerous microorganisms are constantly sequenced, and bioinformatic analysis continuously reveals a high number of biosynthetic pathways for the production of secondary metabolites, whereas only a few natural products can currently be correlated to the genome-sequenced strains. This discrepancy between the genetic capacity for secondary metabolite biosynthesis and low numbers of known compounds has fuelled the development of strategies aimed at the assignment of new secondary metabolites to the predicted genome-encoded pathways. Genome-mining needs, metabolome

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mining, and thus secondary metabolomics—inspired methods are of utmost importance for the success of genomics-based discovery of novel secondary metabolites. Cyanobacteria are a rich source of natural products comprising of primary and secondary metabolites including nonribosomal proteins, polyketides and terpenes and alkaloids, and several of these are known to have anticancer, antiviral, UV protective activities as well as hepatotoxicity and neurotoxicity (Herrero and Flores 2008). Many of the cyanobacterial secondary metabolites have been characterized and are known to be produced via the nonribosomal peptide synthetases (NRPS)/polyketide synthases (PKS) route (Hoffmann et al. 2003). However, several other non-NRPS/PKS synthetic pathways leading to the production of secondary metabolites with the active participation of cytochrome P450 (CYP) are just beginning to be appreciated, with the discovery of many CYP coding genes in their genome.

Many bioactive metabolites produced by cyanobacteria are either a peptide or a macrolide structure, or a combination of both types (Welker and Von Döhren 2006). Other metabolites belong to the alkaloid class of compounds. Two types of biosynthetic pathways produce the peptide class: by giant multidomain enzymes, the NRPS or by ribosomal synthesis and subsequent post-translational modification and processing. NRPS consist of modules, each being responsible for the incorporation of a single amino acid. The order of these modules typically follows a colinearity rule, i.e., the succession of modules corresponds to the order of amino acids in the final product. A minimal module is composed of an amino acid-activating adenylation (A) domain, a peptidyl carrier proteins (PCP) domain carrying the phosphopantetheine cofactor, and a condensation (C) domain. NRPS can accept about 300 proteinogenic and nonproteinogenic substrates and may contain further domains introducing tailoring modifications or epimerizing the amino acid substrates (Grünewald and Marahiel 2006). In contrast, ribosomal biosynthesis of peptides is limited to 20 proteinogenic amino acids. This group of peptides nevertheless displays a high diversity and a considerable biosynthetic and bioactive potential. The ribosomal pre-peptides are typically composed of a leader peptide and core peptide. Associated post-translational modification enzymes (PTMs) catalyze different types of macrocyclizations of the core peptide and side chain modifications of amino acids. Peptide maturation further requires cleavage of the leader peptide by processing proteases (PP) frequently combined with transport across the plasma membrane (Oman and Donk 2010). Macrolides in cyanobacteria are produced by modular type PKS resembling NRPS with respect to their modular nature. In contrast to the peptide-synthesizing enzymes, in PKSs different types of carboxylic acids are activated, assembled, and optionally modified. The maximal set of domains of an individual PKS module is identical to animal fatty acid synthase (FAS) (Jenke-Kodama et al. 2005) and consists of ketosynthase (KS), acyltransferase (AT), ketoreductase (KR), dehydratase (DH), enoyl reductase (ER) and acyl carrier protein (ACP) domains (Staunton and Weissman 2001). Parts of the domains (KR, DH, ER) are optionally used leading to a different reduction state of the keto groups of polyketides. There are also alternative PKS assembly lines cooperating with AT domains encoded in trans of the multi enzymes (Piel 2010), or PKS types comprising single modules that work iteratively (Campbell and Vederas 2010).

The major trait of cyanobacterial pathways is their hybrid character, i.e., the frequent mixture of NRPS and PKS modules. The first biosynthetic pathway identified and partially characterized for cyanobacteria was the mixed NRPS/PKS pathway catalyzing the formation of the hepatotoxin microcystin in the cyanobacterium *Microcystis aeruginosa* (Tillett et al. 2000). The pentapeptide nodularin is structurally closely related and shares a highly similar biosynthetic pathway (Moffitt and Neilan 2004). The anabaenopeptilide pathway in the strain *Anabaena* 90 was described soon after first reports about microcystin biosynthesis. Anabaenopeptilides belong to the cyanopeptolin family of depsipeptides that were shown to inhibit different types of serine proteases (Welker and von Döhren 2006). The signature of this group is the unusual 3-amino-6-hydroxy-2-piperidone moiety (Ahp). The corresponding NRPS assembly line consists of seven modules (Rouhiainen et al. 2000). Unique features of anabaenopeptilides include an integrated formyl transferase domain in the initiation module and nicotinamide adenine dinucleotide dependent (NAD-dependent) halogenase. Aeruginosins are specific inhibitors of serine type proteases and produced by different genera of freshwater cyanobacteria. *Planktothrix agardhii* produces glycosylated variants of the peptides, aeruginosides, via a mixed NRPS/PKS pathway. The signature of this group is the 2-carboxy-6-hydroxyoctahy-droindole (Choi) moiety. The loading module was predicted to activate phenylpyruvate, which is reduced by an integrated KR domain to phenyllactate. Several NRPS/PKS assembly lines were identified and partially characterized for the marine cyanobacterium *Lyngbya majuscula* (Chang et al. 2002). The first pathway described was the biosynthesis of barbamide, a chlorinated lipopeptide with potent molluscidal activity. The lipopeptide contains a unique trichloroleucyl starter unit that is halogenated by unique biochemical mechanisms through the two nonheme iron (II)-dependent halogenases BarB1 and BarB2. Further extraordinary features of the pathway include one-carbon truncation during chain elongation, *E*-double bond formation, and thiazole ring formation. The other important secondary metabolites produced by NRPS/PKS pathways are aeruginosin, cylindrospermopsin, anatoxin, jamaicamide, curacin A, hectochlorin, lyngbyatoxin, apratoxin, nostopeptolide, nostocyclopeptide, cryptophycin.

The majority of cyanobacterial peptides are produced nonribosomally, specifically two peptide families, namely patellamides and microviridins, for which no NRPS pathway could be assigned. Genome-scale analyses have unrivaled further peptide families. Cyanobacteria can now be considered as one of the most prolific sources of ribosomal-produced natural products. The first ribosomal pathway discovered was the biosynthesis of patellamides in the symbiotic cyanobacterium *Prochloron*. The cyclic octapeptides are pseudosymmetric and contain thiazole and oxazolin rings. Patellamides are typically moderately cytotoxic, and some variants were further reported to reverse multidrug resistance (Schmidt et al. 2005). Microviridins are a group of tricyclic depsipeptides predominantly detected in bloom-forming freshwater cyanobacteria. Several members of the family potently inhibit various serine-type proteases. The biosynthetic pathway of microviridins was described for the genera *Microcystis* and *Planktothrix* (Ziemert et al. 2008). Posttranslational modification of microviridins is achieved by the activity of two

closely related adenosine triphosphate (ATP) grasp ligases, MdnB and MdnC. The enzymes introduce two ω-ester linkages between threonine and aspartate and serine and glutamate (MdnC/MvdD) and one ω-amide linkage between lysine and aspartate (MdnB/MvdC). Cyclizations occur in a strictly defined order. Ring size and composition of the microviridin core peptide is invariant (Philmus et al. 2009), whereas N-terminal and C-terminal amino acids are highly variant (Ziemert et al. 2010). The enzyme system further contains a GNAT-type *N*-acetyltransferase and an ABC transporter.

Cyanobacteria produces two types of sunscreen compounds induced under UV irradiation: Scytonemin and mycosporine-like amino acids. Biosynthesis of the two groups of compounds has recently been elucidated, providing further examples for the fascinating natural product biochemistry of cyanobacteria. A gene cluster responsible for scytonemin biosynthesis was initially discovered by random mutagenesis in the terrestrial symbiotic cyanobacterium *Nostoc punctiforme* (Soule et al. 2007). The gene cluster contains a number of genes related to aromatic amino acid biosynthesis (Soule et al. 2007). The biosynthetic route was proposed to start with tryptophan and tyrosine. Two of the initial steps of the sunscreen synthesis were reproduced *in vitro* (Balskus and Walsh 2008). The open reading frames (ORF) NpR1275 was confirmed to act as a tryptophan dehydrogenase, whereas *p*-hydroxyphenylpyruvic acid was proposed to be generated by the putative prephenate dehydrogenase NpR1269. Both substrates are then further transformed by the thiamin diphosphate (ThDP)-dependent enzyme NpR1276 to isomeric acyloins representing one-half of the carbon frameworks of scytonemin (Balskus and Walsh 2008). The enzyme showed a remarkable selectivity for the specific C–C bond reaction that is unprecedented in natural systems. The other sunscreen compound microsporines consist of a single amino acid linked to cyclohexenone. Cyanobacteria and other algae produce mycosporine-like amino acids, which contain two substituents linked to the central ring by imine linkages. Four enzymes are involved in the synthesis of the specific MAA (mycosporine-like amino acid) shinorine in *Anabaena vari-abilis* ATCC 29413: a dehydroquinase synthase homologue (DHQS), an *O*-methyl-transferase (O-MT), an ATP grasp ligase, and an NRPS-like enzyme.

There is considerable evidence that some stress responses can be used to trigger the expression of secondary metabolic genes. Altering a single parameter in the growth conditions and eliciting a stress response has been previously applied through the one strain many compounds (OSMAC) approach to explore the secondary metabolic potential of different strains of cyanobacteria (Edwards and Ericsson 1999). Cytochrome P450s CYPs are a group of ubiquitous hemoprotein oxygenases that are found in all domains of life (Nebert et al. 1989). The great diversity, occurrence, and distribution of CYP suggest that they could be involved in essential or crucial metabolism, such as defense against environmental pollutants, drug detoxification, synthesis of important molecules, and defense against extreme environmental (Bernhardt 2006). Several CYP and CYP-like genes have been identified in cyanobacterial genomes, however, little attention has been given to their functional characterization (Ke et al. 2005; Kühnel et al. 2008; Alder et al. 2009). Elucidation of the crystal structure of CYP120A1 has shown that it participates

in retinoid metabolism (Ke et al. 2005). A BLASTp search in the National Center for Biotechnology Information (NCBI) cyanobacterial genome database using the characterized protein NP_488726 from *Nostoc* sp. strain PCC 7120 (Agger et al. 2008) or CYP120A1 from *Synechocystis* sp. PCC 6803 (Kühnel et al. 2008) yields 100 putative CYP sequences and it distributed in most of the known cyanobacterial species. CYP110 from *Nostoc* sp. PCC 7120 is not a cytosolic enzyme, but membrane bound, like eukaryotic CYPs. It is not induced by alkanes, and does not participate in alkane biodegradation, but it is involved in v-hydroxylation of long chain fatty acids and plays a role in nitrogen fixation (Torres et al. 2005). Isoprenoids are one of the major structural classes of natural products. Recent efforts have shown that cyanobacterial strains are capable of producing isoprenoids (Agger et al. 2008). In addition, marine cyanobacteria from *Microcoleus* and *Phormidium* genera have been known to contribute to bioremediation of oil spills using CYP as a catalyst for the alkane breakdown (Hasan et al. 1994). Though cyanobacteria are important as a source of natural product the CYP related pathways are only recently becoming apparent. The increasing number of functionally characterized CYPs from cyanobacteria, as well as evidence of terpene synthase genes, is opening new vistas of natural product formation (Agger et al. 2008).

While some studies attempted the activation of biosynthetic pathways which were assumed as being "silent" under standard laboratory conditions, through the variation of cultivation methods, by introducing environmental challenges or by modifying regulatory mechanisms (Hertweck 2009) others aimed to provide evidence at the transcriptomic and proteomic level to help the identification of active biosynthetic pathways and subsequently uncover the corresponding metabolites (Schley et al. 2006). As a straightforward method to directly assign novel metabolites to predicted biosynthetic pathways, the construction of knockout mutants by targeted gene inactivation coupled to comparative metabolite profiling has been used successfully to disclose previously undiscovered secondary metabolites (Cortina et al. 2012). Furthermore, nowadays a variety of methods have been developed that follow a holistic "metabolites first" principle, i.e., all metabolites in a given analytical method are able to detect are first recorded, and using of hyphenated high-performance liquid chromatography in combination with high-resolution tandem mass spectrometry (LC-HRMS), and matrix assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) were analyzed in a high throughput scheme.

2.2 Resent Advances in Novel Secondary Metabolites Discovery from Cyanobacteria

With advances in analytical techniques, there is a tremendous increase in discoveries of secondary metabolites. Low cost, chemical free green extraction (GE) methods such as supercritical fluid extraction (SFE), pressurized liquid extraction (PLE) are now gaining popularity for extraction of secondary metabolites. Analytical

techniques like advanced ultra-performance liquid chromatography (UPLC), which can be a better option than high-performance liquid chromatography (HPLC). Many cyanobacteria can be cultured as single-species communities. The microbial communities, or colonies, curate their environment via metabolic exchange factors such as released natural products. To date, there are very few tools available that can monitor, in a systematic and informative fashion, the metabolic release patterns by microbes grown in a pure or mixed culture. There are significant challenges in the ability to monitor the metabolic secretome from growing microbial colonies. For example, the interactions of such molecules can be extremely diverse, ranging from polyketides, nonribosomal peptides, isoprenoids, fatty acids, and microcins to peptides, poly-nucleotides, and proteins (Cane 2010). Because of this chemical diversity, most of these molecules are extracted prior to analysis and studied one at a time and apart from the native spatial context of a microbial colony. Thus, limited information is obtained about the metabolic output of colonies in a synergistic or multiplexed fashion. Genomics is the most prominent tool to define the makeup and species dynamics of the microbiome. Once the complete genome of an organism is sequenced, genome-mining approaches can be used to predict natural products and to discover novel adaptive metabolites. Generation of annotated genomes helps to provide a basis for predictive algorithms capable of mining unannotated genomes for new secondary metabolites, which can drastically improve the speed at which new molecules can be targeted and characterized. We discuss here recent advances in the extraction of novel secondary metabolites from cyanobacteria.

2.2.1 Epigenetic-related Approaches

Secondary metabolites in cyanobacteria confer an evolutionary benefit to the producing organism. In the simplified environment of the laboratory, cyanobacteria often do not depend on the entire capabilities of their secondary metabolome and thus the products of most of the biosynthetic gene clusters are not observed. Improvements in *de novo* genome sequence technologies have resulted in a dramatic increase in the number of complete genomes available for well-known producers of natural products. These data have revealed that many members of these groups produce only a small fraction of the natural products encoded in their genomes under standard laboratory conditions. The natural product biosynthetic pathways that are not expressed, often referred to as the "silent metabolome," therefore, potentially represent a vast reservoir of undiscovered small molecules. Epigenetic enzymes like histone deacetylases (HDACs) and DNA methyltransferases (DNMTs) play a crucial role in gene regulation of biosynthesis clusters (Schmitt et al. 2011). Recently, it was discovered that interference with these systems can result in upregulation of biosynthetic clusters (Cichewicz 2010). In chemical epigenetics, HDAC and DNMT inhibitors are used to manipulate the epigenome. Similar effects can be obtained through genetic manipulation of genes encoding global transcriptional regulators like LaeA (Bok et al. 2006), histone deacetylases (Lee et al. 2009), or

methylating enzymes (Bok et al. 2009), as well as the small ubiquitin-like modifier (SUMO) protein (Szewczyk et al. 2008). The complexity of the affected systems is demonstrated by the fact that, in all cases, gene deletion and over expression increased the expression of some biosynthetic clusters and decreased the expression of others. In this context, epigenetic approach is a novel tool to get valuable metabolites from cyanobacteria.

2.2.2 Genome-Driven Approaches

Genome mining as an approach to natural product discovery has recently been studied (Gross 2009; Velasquez andDonk 2011). While it is generally possible to identify the biosynthetic gene cluster for a known compound produced by a microorganism from genome sequence data, the converse approach of predicting the exact structure of a natural product from sequence data is often not possible. Several factors contribute to this problem, including difficult to predict post-assembly, modification, biosynthetic domain skipping, ambiguous cyclization patterns, and noncolinearity of some biosynthetic enzymes. Although bioinformatics tools exist to analyze genome data, to identify natural product biosynthetic clusters with a low level of accuracy, to predict the structure of the encoded compound, there is room for significant advancement in this field. There are possibility to identify silent gene clusters in natural product producing microorganisms by subtractive analysis, comparing the observed compounds to biosynthetic pathways predicted using existing bioinformatics tools (Bachmann and Ravel 2009; Schmitt et al. 2011). Annotated genome sequence data can facilitate directed genetic strategies aimed at de-silencing individual gene clusters. Positive regulators are often colocalized with the biosynthetic pathways under their control. Integration of a functional promoter in front of these regulators has the potential to upregulate the entire biosynthetic pathway. This approach requires a method to introduce DNA into the genome of the microorganism via homologous recombination, as well as rudimentary genetic tools including a validated promoter and a suitable selectable marker. The same strategy can be used to integrate a functional promoter in front of a silent biosynthetic pathway; although this strategy may only be practical when a single transcript is predicted to contain all of the necessary genes for production of the compound. Expression of silent biosynthetic pathways in heterologous hosts offers another strategy for de-silencing biosynthetic pathways. The emergence of phage protein based recombining approaches has significantly aided in manipulating large DNA fragments and has made heterologous expression of natural product pathways a viable option (Thomason et al. 2007). Heterologous expression of pathways cloned directly from DNA isolated from environmental samples (a culture independent approach) for accessing natural product chemical diversity, is under explored (Feng et al. 2010). Currently, heterologous expression is a very labor-intensive strategy that limits its broad application in the drug discovery process; however, when a biosynthetic

pathway of high interest has been identified this approach offers an additional tool to study the pathway in a genetically tractable host.

Genome mining has long been used to predict molecular structures (Liu et al. 2010) and is still used to successfully characterize novel NRPS, PKS, terpenoid, and other natural products. Genome-mining searches for genes or gene clusters that encode enzymes involved in the biosynthesis of natural products are based on sequence alignment with other characterized enzymes involved in that specific natural product biosynthesis. Genome-screening programs such as ClustScan, NRPS-PKS, and "NP. Searcher" are used to predict locations of gene clusters and the structure of their putative products (Challis 2008). Success of genome mining depends on the availability of complete microbial genomes, thus will prove especially powerful when used in conjunction with metagenomics, as sequenced genomes and plasmids can be automatically fed into a pipeline to be mined for secondary metabolite production (Challis 2008). The tools currently in place provide a good starting point for data mining; however, improvements are still needed in predictive software for adaptive metabolites, including a ribosomal encoded peptide predictor, incorporation of 6-frame translation into genome-mining searches, and consolidation of metabolite and small molecule databases.

2.2.3 Analytics and Preparative Strategies for Accessing New Natural Products

Though many tools contribute to "-omics" studies and natural product workflows, mass spectrometry (MS) and nuclear magnetic resonance (NMR) are the two most powerful tools. They are already used for individual "-omics" approaches. MS is capable of high throughput identification of proteins, metabolites, and other types of molecules through the generation of important structural information with tandem MS. Like MS, NMR is often used for compound identification as well as to observe global metabolite changes. NMR is able to provide structural information, atomic connectivity, and stereochemistry that MS cannot. Ongoing improvements of these two technologies have allowed for optimal data generation from a small sample size or from crude samples. Nanomolar NMR elucidates structures from as little as one nano mole of material (Molinski 2010), although it is anticipated that this will be in the picomolar range in the near future, while some MS methods can evaluate samples in the sub-picomolar range. NMR of crude mixtures has been previously used for a variety of samples and is advantageous since it eliminates the need to separate molecules prior to analysis (Exarchou et al. 2005; Schroder et al. 1998). Combining liquid chromatography (LC) with MS or NMR allows for the separation of molecules preceding analysis, resulting in improved signal intensity. Additionally, spatial localization of molecules can be characterized using IMS (Imaging mass spectrometry) (Cornett et al. 2007), often giving important information about signal distribution in the cell (Seeley and Caprioli 2008) or in interactions grown on agar (Yang et al. 2009). Each tool has an invaluable role in "-omics" studies and has

great potential in advancing microbiome-based workflows in studies that will both catalog known molecules and target uncharacterized molecules.

A breakthrough in natural product analytics has been the introduction of ultra-high pressure liquid chromatography (UHPLC) (Wolfender et al. 2010). In combination with time-of-flight (TOF) MS detectors with very fast response times, UHPLC is a very efficient tool for de-replication of natural product extracts and for genome-driven identification of new natural products (Grata et al. 2008). The excellent sensitivity and resolution of contemporary chromatography systems enables the miniaturization of the entire process of broth screening making it much more powerful and less cumbersome than in the past. In particular, principal component analysis (PCA) has become increasingly popular. The main concept of PCA is to reduce a large dataset, obtained for example from different LC–MS experiments, in order to extract the most important variations between the samples without significant loss of information (Kuhnert et al. 2011). For structure elucidation of these compounds, LC–NMR spectroscopy can be used. As on-flow or stop-flow LC–NMR methods having inherently low sensitivity and are limited to the measurement of proton NMR spectra, SPE-NMR (trapping of the LC-flow on a SPE [Solid-phase extraction] column) or CapNMR (micro fractionation followed by measurement of a concentrated sample via micro-flow capillary LC–NMR probes) can overcome these limitations and can be used for structure elucidation through 1D and 2D measurements (Wolfender et al. 2010). For the purification of natural products, supercritical fluid chromatography (SFC) is becoming a powerful tool. While this technology was introduced nearly 50 years ago, only in recent years have many of the instrumental limitations been overcome. In combination with MS detection, new natural products can be isolated very efficiently using MS-guided fractionation (Guiochon and Tarafder 2011). All these technical innovations have reshaped natural product chemistry significantly over the past couple of years. The reisolation of known secondary metabolites could be avoided early in the process using high-resolution analytical tools. The bothersome purification of natural products can be performed semiautomatically and much more efficiently with the current instrumentation. As a consequence of these technical improvements the costs dropped to generate pure natural product libraries.

The relatively rapid execution and chemically specific information provided by MALDI IMS experiments offers an enticing tool to enhance the preclinical trials of pharmaceutical compounds (Gessel et al. 2014). For a targeted approach, using the known molecular mass of a compound offers a distinct advantage in defining its localization, allowing experimenters to easily distinguish the target from unknown species and/or electronic and chemical noise. Proteomic changes arising from treatment with a specific pharmaceutical compound can be tracked in conjunction with the distribution and metabolism of the compound. Imaging of pharmaceutical compounds and their metabolites is quite challenging because of the small size of the drug molecules, but with high resolution mass analyzers these species can be successfully isolated in the lower mass regions (100–500 Da) (Chughtai and Heeren 2010). In a recent study Shanta and colleagues addressed the issue of matrix interference in low mass regions and drug/drug metabolite detection via MALDI IMS

(Shanta et al. 2012). They detail the development of a binary matrix compound that has significantly reduced interference peaks from 0 to 500 Da. IMS is a powerful tool for simultaneously investigating the spatial distribution of multiple different biological molecules. The technique offers a molecular view of the peptides, proteins, polymers, and lipids produced by a microbial colony without the need for exogenous labels or radioactive trace material (Gonzalez et al. 2012). Target compounds can be measured and visualized simultaneously and in a high throughput manner within a single experiment. IMS extends beyond techniques such as MALDI profiling or MALDI intact cell analysis. Although invaluable, these techniques give a broad view of the metabolites produced in reference to a growing colony, where discretely secreted low global concentration but high local concentration metabolites could be missed. IMS entails examining the entire cyanobacterial colony, including the surrounding agar medium, by defining a raster composed of greater than 1000 laser spots (points of data collection), which increases the likelihood of detecting unique, discrete ion distribution patterns, and hidden molecular phenotypes that cannot be observed by the naked eye. Using MALDI-TOF, several new cyanopeptides were observed and characterized. Recently, MALDI-TOF was used to directly analyze cyanobacteria for the presence of cyanopeptides and toxins from 850 individual colonies (Welker et al. 2006). In this study, a small colony of the cyanobacterium was placed on a MALDI-TOF-plate and covered with a small amount of 2,5-dihydroxybenzoic acid matrix before they were analyzed by MALDI-TOF mass spectrometry. A total of 90 individual peptides was identified from these 850 individual cyanobacteria colonies, including 18 that appear to be unique from their masses. Erhard et al. (1997) used MALDI-TOF MS for identification of secondary metabolites with intact cyanobacterial cells. Resulting mass signals, which are further characterized by post source-decay fragmentation, and comparison of observed fragment spectra with theoretical ones or with those of pure reference compounds. Desorption electrospray ionization mass spectrometry (DESI-MS) is another applied analytical technique for chemical profiling, characterization and quantification of low molecular weight biomolecules (Esquenazi et al. 2008). Another technique is a direct analysis in real-time mass spectrometry (DART-MS), which is very much effective in chemical profiling and fingerprinting of bioactive molecules without prior sample preparation. Singh and Verma (2012) have identified the *Nostoc* sp. on the basis of characteristic chemical compounds (chemical finger printing) using DART-MS. Advances in all these analytical techniques have a tremendous impact on the identification and characterization of natural products and hope many new bioactive natural products will come up with advances in this area.

2.3 *In silico* Screening for Novel Secondary Metabolites of Cyanobacteria

Traditionally, drug-target discovery is fundamentally a wet lab experimental process comprising of identification of candidate lead compounds in chemical/activity-guided screening programs, where extracts and purified compounds are tested

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