Genomics driven metabolomics novel strategies for the discovery and identification of secondary metabolites

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Chapter 1

General Introduction

General introduction

Metabolic Pathway Analysis: Basic concepts and scientific applications in the post-genomic era

Natural products have been of major interest due to their pharmaceutically exploitable properties like antibacterial (Christophersen, et al., 1998), antifungal (Coleman, et al., 2011), antiparasitic (Wink, 2012), anticancer (Nirmala, et al., 2011) or immunosuppressive (von Wartburg and Traber, 1988) activities. Although not always used directly as a drug they act as a reliable source for novel and innovative therapeutic agents offering valuable chemical scaffolds for novel drugs (Newman and Cragg, 2007). The primary source of these structurally heterogenic molecules are natural sources like bacteria and fungi, which is not surprising as these microorganisms live in complex ecosystems where they compete and communicate with other organisms (Goh, et al., 2002; Losada, et al., 2009) Up till today, natural products represent the largest source of drugs offering novel chemical entities. Although newer techniques like combinatorial chemistry have been used as a discovery source for several years, solely one approved drug with a new chemical entity was reported to be discovered between 1982 and 2007 using this method (Newman and Cragg, 2007). In 2005, Bayer's antitumor compound sorafenib, obtained from combinatorial chemistry, was approved by the FDA. This gives reason to expect that microorganisms will play also a major role in future drug developments.

However, the incredible increase in the amount of sequencing data, primarily driven by the falling cost of DNA sequencing, has led to a paradigm shift in the approach of secondary metabolite discovery (Letzel, et al., 2013). The analysis of the growing number of sequenced fungal genomes revealed that most of the genes responsible for secondary metabolite production are located in clusters which far outweigh the number of described secondary metabolites (Brakhage and Schroeckh, 2011). That means, that despite the discovery of countless natural products during the last decades a vast number of novel natural products with potent bioactivities based on novel structural features still awaits discovery. With the use of genome sequencing data unknown secondary metabolite clusters can be identified without a priori knowledge of a strain's ability to produce natural products. This approach of secondary metabolite discovery is known as genome mining and has been successfully applied for the identification of various novel metabolites (Letzel, et al., 2013).

These metabolites are produced by large, multifunctional protein complexes called non ribosomal peptide synthetases (NRPS) or polyketide synthetases (PKS) which catalyze the stepwise condensation of simple amino acids or malonyl building blocks to complex molecules. Although their substrates can differ considerably, these multi-modular enzymes show striking similarities in their architecture as well as in the mechanisms used for product assembly.

NRPSs have a modular organization, with each module responsible for one or more chain-elongation step. Every single module is subdivided into three basic domains that carry all essential information for recognition, activation and modification of one substrate. At a minimum, a typical NRPS module consists of an adenylation (A) domain responsible for amino acid activation, a thiolation (T) domain, also

known as peptidyl carrier protein (PCP), which binds the activated amino acid and a condensation (C) domain that catalyzes peptide-bond formation. The common arrangements of these domains follow a $(C\text{-}A\text{-}PCP)_n$ organization. Additionally, a variety of optional domains have been described (Schwarzer, et al., 2003) such as methyltransferase (MT) and epimerization (E) domains (Figure 1).

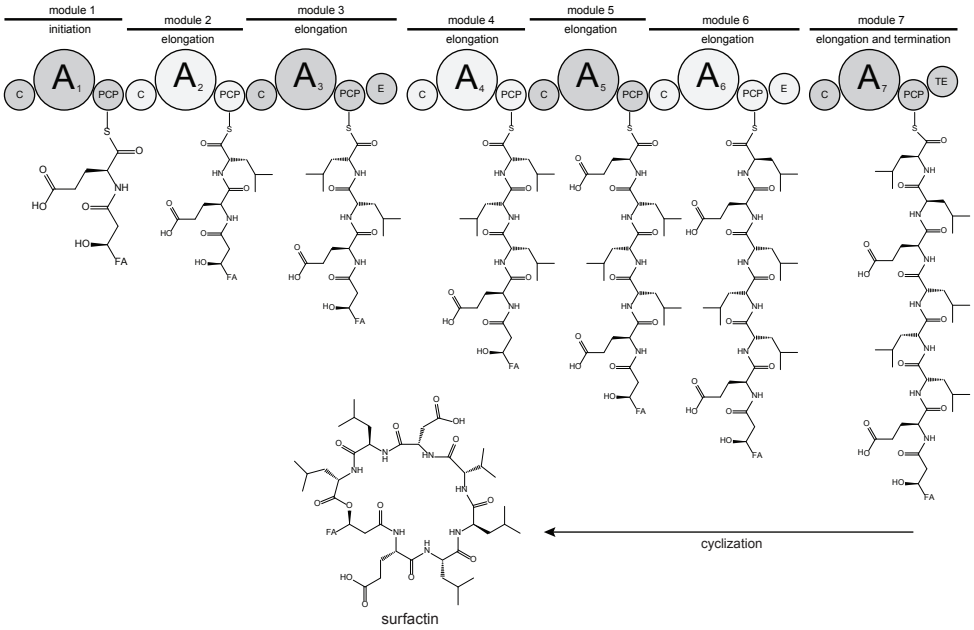

Figure 1. NRPS assembly of surfactin
The surfactin biosynthesis consists of seven modules each responsible for one chain-elonging step. Each module is divided in various domains catalyzing the activation (A domain), covalent binding (PCP domain), elongation (C domain), epimerization (E domain) and termination by covalent cyclization (TE domain).

A similar domain architecture can be found in PKS systems with an acyltransferase (AT) domain responsible for building block selection and transfer, an acyl carrier protein (ACP) for unit loading and a ketosynthase (KS) domain for decarboxylative condensation of the extending substrate. The resulting keto thioester can subsequently be modified by ketoreductase (KR) domains, dehydratase (DH) domains and enoyl reductase (ER) domains (Cane, 1997; Staunton and Weissman, 2001). In addition, modular NRPS and PKS systems can closely cooperate to form a third group of gene clusters, so-called hybrid products.
The number of modules and their domain organization within the enzymes control the structures of the final products (Grunewald and Marahiel, 2006; Schwarzer, et al., 2003; Schwarzer, et al., 2002). Thus, the order of modules usually corresponds to the sequence of building blocks in the final product. Many systems adhere to this mechanistic paradigm, which is often referred to as the “co-linearity rule” (Fischbach and Walsh, 2006). However, several exceptions to this rule have been discovered in

the last years including iterative NRPS and PKS which incorporate multiple residues of the same unit iteratively into the final structure and the so called nonlinear NRPSs which deviate completely from the standard domain organization leading to unexpected products (Mootz, et al., 2002; Shaw-Reid, et al., 1999; Shen, 2003). In 2008, the genome of Penicillium chrysogenum Wisconsin54-1255 was sequenced revealing 20 polyketide synthases (PKS), 10 nonribosomal peptide synthetases (NRPS) and 2 hybrid NRPS-PKS genes clusters (van den Berg, 2008). According to the deduced domain organization up to 20 polyketides, 10 nonribosomal peptides and two products with mixed properties are expected. However, most gene clusters encoding biosynthetic systems could not be associated with the production of a known metabolite and are therefore referred to as 'cryptic' or 'orphan'. The reason for the lack of assignment is that under standard laboratory conditions the majority of secondary metabolite biosynthesis gene clusters is not expressed (van den Berg, 2011). These clusters remain silent as long as the triggers for their induction have not been identified. (Brakhage, et al., 2008). Furthermore, due to the complexity of these clusters almost no products could be directly deciphered from the genetic sequence with sufficient accuracy and confidence (van den Berg, 2011).

Strategies for the discovery of new natural products

Several strategies can be applied to elucidate the function of biosynthetic gene clusters, depending on the expression of latter (Challis, 2008). For expressed cryptic genes inactivation of a biosynthetic gene, which is presumed to be essential within the biosynthetic gene cluster, followed by comparative metabolite profiling of the wild-type organism and the non-producing mutant is the standard approach for defining the functions of genes and for the discovery of novel natural products (Brakhage and Schroeckh, 2011) (Figure 2). The metabolites present in the wild-type but missing in the mutant are likely to be products of the cryptic gene cluster and can be isolated and structurally characterized. (Figure 2) Alternative strategies are applied when the gene cluster of interest is silent involving promoter exchange, overexpression of transcription factors or other pleiotropic regulators. (Brakhage and Schroeckh, 2011; Chiang, et al., 2011; Schumann and Hertweck, 2006)

All approaches combine an elaborate and refined strategy to discover, isolate, and characterize novel natural products. They all have in common that sensitive, accurate, quantitative and fast analytical techniques are required to detect differences in production related to the conducted genetic modification. Furthermore, due to the rapid advances in DNA sequencing technology it is conceivable that, in the recent future, thousands of cryptic natural product biosynthetic gene clusters will become available, leaving the discovery of the metabolic products of these clusters the bottleneck (Zerikly and Challis, 2009).

Metabolite discovery and metabolomics

The aim of metabolomics is to measure the full metabolome, which consists of all low molecular weight species in cells, tissues, organs or organisms (Griffin, 2004). Unlike the proteome, which represents the entire set of proteins, primarily build from 20 basic amino acids with a limited set of modifications and a rather defined distribution of physicochemical properties, are metabolites a highly diverse range of compounds differing in mass, size and polarity. With concentrations ranging

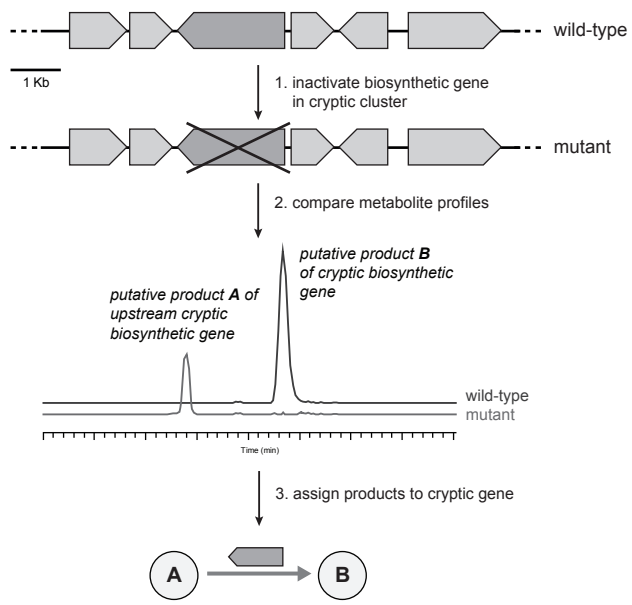

Figure 2. Principle of a gene knockout strategy in combination with comparative metabolite profiling to elucidate the role of a cryptic biosynthetic gene.

over several orders of magnitude (pM – mM) combined with often a short lifetime represent metabolites a challenging class of compounds to analyze. Different from transcriptomics and genomics, which utilize polymerase chain reaction (PCR) to amplify DNA sequences, there is no effective tool to unbiasedly increase the concentration of all low abundant metabolites. Therefore, more than one analytical method is required for the acquisition of a comprehensive metabolite profile attempting to describe the global metabolism (van der Greef, et al., 2007). Even today, despite the rapid advances in technical instrumentation in the last years, no analytical tool is capable to completely cover the entire metabolome of even the simplest organism (Heather, et al., 2013).

The two most commonly used techniques in metabolomics are NMR spectroscopy and mass spectrometry. NMR is based on the measurement of isotopes that contain an odd number of protons and/or neutrons in a magnetic field. Typically, metabolites higher concentrated than 5-10 µM can be detected if they are not co-resonant with higher concentrated metabolites. (Heather, et al., 2013). Despite this limited sensitivity (Martin, et al., 2007), NMR spectroscopy is widely used in metabolomics studies due to its speed, non-invasiveness and robustness and its highly quantitative nature. As the area of a peak is proportional to the concentration of the metabolite measured, determination of absolute concentrations is possible.

In contrast, mass spectrometry (MS) techniques which measure molecules as ions in the gas phase after ionization, are fast and highly sensitive with a large dynamic range. By fragmenting these ions, structural information can be obtained supporting the identification of relevant metabolites (Dettmer, et al., 2007). As a wide range of different MS instruments are available, ranging from high resolution MS such as Fourier transform ion cyclotron resonance (FTICR), Orbitrap FT and time-of-light

(TOF) to low resolution MS (ion traps, triple and single quads) up to hybrid systems, various techniques with different emphasis can be applied.
However, mass spectrometry is most commonly coupled to chromatographic separation techniques like gas chromatography (GC-MS) and liquid chromatography (LC-MS) allowing the separation of complex mixtures into its individual components. By doing so, LC-MS and GC-MS exceed the sensitivity of NMR spectroscopy by many orders of magnitude. For these reasons, mass spectrometry is the primarily used technique in metabolomics studies currently outnumbering the application of NMR (Dettmer, et al., 2007).
For GC–MS, metabolites have to be volatile or made volatile using derivatization techniques. Especially small molecules like amino acids, sugars, glycolytic intermediates, fatty acids and TCA cycle intermediates can be detected after derivatization using derivatization reagents like N-methyltrimethylsilyltrifluoroacetamide (MSTFA). The most common ionization technique in GC-MS is electron ionization (EI) which produces, next to the radical molecule cation, characteristic fragments for many metabolites allowing their identification with databases like NIST or the Golm Metabolome Database (Kopka, et al., 2005). While a robust and versatile technique, GC–MS is limited to compounds which are or can be made volatile and thermostable enough for application in the system. In cases where this cannot be achieved, LC–MS is an option which in principle does not require derivatization, allowing fast analysis.
Depending on the polarity of the analytes of interest, a suitable separation technique like normal-phase liquid chromatography (NPLC), reversed-phase liquid chromatography (RPLC) or hydrophilic interaction chromatography (HILIC) (Tolstikov and Fiehn, 2002) can be chosen for an efficient separation of the individual compounds. After chromatographic separation, the analytes are introduced into the mass spectrometer using an ionization technique like electrospray ionization (ESI) or less frequent atmospheric pressure chemical ionization (APCI) (Niessen, 2003). As minimal fragmentation takes place during ionization, the measured mass of an analyte, recorded with high mass accuracy and resolution, is expected to be close to the anticipated mass of a specific metabolite recorded in a publically accessible database which potentially allows a direct putative identification. Several databases containing metabolite information are available in order to facilitate putative annotation of accurate mass signals. (e.g. PubChem; HMDB; KEGG, KNapSAcK, MZedDB).

Metabolite Profiling
Metabolomics experiments can be conducted as non-targeted profiling using an unbiased approach which aims to measure as many metabolites as possible. As the conditions are not optimized for a particular set of compounds, limits of detection can be severely impaired resulting, at worst, in a failure to detect and identify the desired metabolites.
If more information about the metabolites of interest is available, more targeted profiling approaches can be used focusing on a particular class of compounds. As the biosynthesis of secondary metabolites by PKS or NRPS systems underlays modular based mechanisms, bioinformatics tools can be used to predict substrates, their subsequent reactions and potential products (Figure 3). The calculation of their

corresponding physiochemical properties facilitates the subsequent discovery of these natural products from biological origins as an appropriate targeted profiling method, optimized for their detection, can be selected. Often a non-targeted profiling approach yielding semi-quantitative data is followed by a targeted profiling

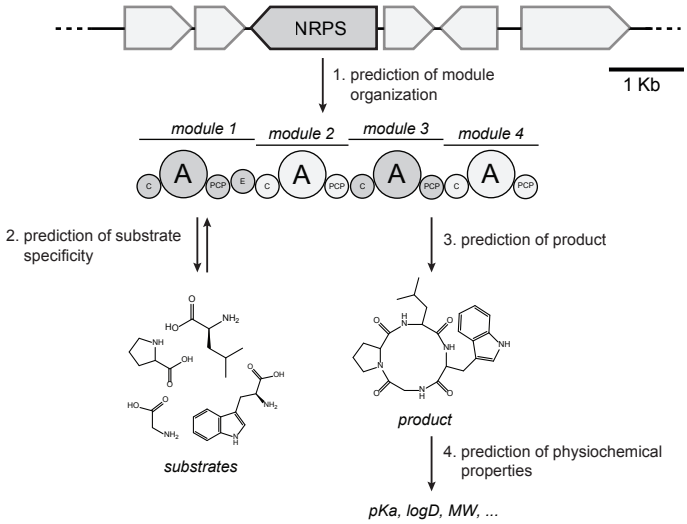

Figure 3. Bioinformatics driven selection of an analytical approach for metabolite discovery
Prediction of the modular organization of a cryptic PKS/NRPS system in combination with calculated substrate specificities results in possible products synthesized by the biosynthetic gene cluster. Predicting their physiochemical properties can help to select appropriate analytical techniques for their detection.

method which allows the determination of absolute concentrations. By identifying metabolites varying between wild-type and mutant samples using statistical methods like the Student's t-test, analysis of variance (ANOVA) or principal component analysis (PCA), metabolic changes can be identified which are related to the genetic modification. This approach, called comparative metabolite profiling, is the most often used approach for the determination of metabolic differences (Fernie, et al., 2004).

Structure elucidation in metabolomics

Metabolite identification is an essential part of any metabolomics study and frequently cited as the major bottleneck in metabolomics (Scalbert, et al., 2009). Without a proper identification, the discovery of metabolic alterations related to a biological question is uninterpretable and limited to diagnostic purposes only. However, as one of the most challenging and labor intensive parts in the metabolomics framework, metabolite identification is neglected in many studies or even completely ignored as the distinctive versatility of metabolites, with millions of possible structures even matching a given elemental composition, imposes a major challenge.

Commonly, complementary analytical techniques like NMR, IR, UV, MS, etc. are employed with each demanding different properties from a given sample. Mass

spectrometry in combination with various fragmentation techniques represents one of the most routinely used techniques for metabolite identification as it requires small sample volumes, low sample concentrations and purity and short analysis times. By fragmenting ionized molecules and detect their charged fragments structural information can be obtained. Using this approach in combination with the identified elemental composition of a metabolite, MS/MS databases can be built and screened in order to match an identical database entry (Horai, et al., 2010; Wishart, et al., 2009). However, different levels of identification are defined depending on the available metadata (Sumner, et al., 2007). Whereas on level 1 two independent and orthogonal techniques are required for a full identification of a non-novel metabolite using a reference compound, represents a level 2 identification a putatively annotated compound without chemical reference standards available but identity to metadata from an entry in a database. A level 3 identification is the putatively characterization of a compound based on similarities to a specific class of compounds. Metabolites identified at level 4 represent unknown compounds which can be differentiated and quantified but are structurally not characterized.

Scope of the thesis

The aim of this thesis is to demonstrate how to discover, identify and ultimately assign novel secondary metabolites with new structural features harboring potentially pharmaceutically exploitable properties to their corresponding NRPS and/or PKS gene clusters in Penicillium chrysogenum using genome mining strategies. For this, an adequate analytical pipeline is required which allows the unbiased detection of genetic modifications on a metabolic level. As products and intermediates of secondary metabolite gene clusters show complex chemical structures and are mostly present only at low concentrations, an advanced mass spectrometry based pipeline was developed which enables the identification of structural complex metabolites at low concentrations directly from a biological matrix.

Outline of the thesis

In Chapter 2, the validation and further application of a targeted profiling method is described for the identification of secondary metabolites originating from a cryptic di-modulated NRPS gene cluster. As bioinformatics analysis of the gene cluster predicted various modified dipeptides involved in the biosynthetic pathway, a reversed phase based separation method was developed. After structure elucidation of metabolites using NMR and MS/MS based techniques, detailed enzymatic reaction steps were identified and their corresponding genes assigned.

In Chapter 3, the identification of structurally novel compounds originating from the roquefortine/meleagrin pathway is described and their impact on the previously proposed pathway discussed. As a results, new biosynthetic reactions were found, indicating excessive branching of the previously as linear reported pathway, with several pharmaceutically interesting end products.

The application of the secondary metabolite profiling pipeline for samples derived from genetic modifications of a cryptic tetra-modular NRPS system is subject of Chapter 4. As this non-linear NRPS lacks distinctive substrate specificity, various novel tetrapeptides could be found, harboring similar physiochemical properties,

thus making their discovery and identification challenging. In combination with substrate predictions, a detailed biosynthetic mechanism is proposed.
In Chapter 5, a novel mass spectrometry based structure elucidation pipeline for the de novo structure identification of small molecules, coined CMCP (Chemoinformatics supported MSn Comparison Pipeline) is presented. By transferring fragmentation mechanisms from a similar fragmenting database entry, complete structures could be identified solely using mass spectrometry. To demonstrate the various concepts of fragmentation tree comparison, the de novo identification of structurally complex secondary metabolites, obtained from comparative metabolite profiling from Chapter 2 and 3, is described.
In Chapter 6, the application of CMCP in direct infusion based experiments is demonstrated. Challenges and advantages resulting from the lack of a separation step prior MS analysis are discussed. To demonstrate the capabilities of this approach to cope even with co-fragmenting interferences like isobaric and isomeric ions, the structure elucidation of various complex metabolites from liquid cultures of Penicillium chrysogenum is described using DI-MS in combination with CMCP.

References

Brakhage, A.A., and Schroeckh, V. (2011). Fungal secondary metabolites - strategies to activate silent gene clusters. Fungal Genet Biol 48, 15-22.

Brakhage, A.A., Schuemann, J., Bergmann, S., Scherlach, K., Schroeckh, V., and Hertweck, C. (2008). Activation of fungal silent gene clusters: a new avenue to drug discovery. Prog Drug Res 66, 1, 3-12.

Cane, D.E. (1997). Introduction: Polyketide and Nonribosomal Polypeptide Biosynthesis. From Collie to Coli. Chem Rev 97, 2463-2464.

Challis, G.L. (2008). Mining microbial genomes for new natural products and biosynthetic pathways. Microbiology 154, 1555-1569.

Chiang, Y.M., Chang, S.L., Oakley, B.R., and Wang, C.C. (2011). Recent advances in awakening silent biosynthetic gene clusters and linking orphan clusters to natural products in microorganisms. Curr Opin Chem Biol 15, 137-143.

Christophersen, C., Crescente, O., Frisvad, J.C., Gram, L., Nielsen, J., Nielsen, P.H., and Rahbaek, L. (1998). Antibacterial activity of marine-derived fungi. Mycopathologia 143, 135-138.

Coleman, J.J., Ghosh, S., Okoli, I., and Mylonakis, E. (2011). Antifungal activity of microbial secondary metabolites. PLoS One 6, e25321.

Dettmer, K., Aronov, P.A., and Hammock, B.D. (2007). Mass spectrometry-based metabolomics. Mass Spectrom Rev 26, 51-78.

Fernie, A.R., Trethewey, R.N., Krotzky, A.J., and Willmitzer, L. (2004). Metabolite profiling: from diagnostics to systems biology. Nat Rev Mol Cell Biol 5, 763-769.

Fischbach, M.A., and Walsh, C.T. (2006). Biochemistry. Directing biosynthesis. Science 314, 603-605.

Goh, E.B., Yim, G., Tsui, W., McClure, J., Surette, M.G., and Davies, J. (2002). Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. Proc Natl Acad Sci U S A 99, 17025-17030.

Griffin, J.L. (2004). Metabolic profiles to define the genome: can we hear the phenotypes? Philos Trans R Soc Lond

B Biol Sci 359, 857-871.

Grunewald, J., and Marahiel, M.A. (2006). Chemoenzymatic and template-directed synthesis of bioactive macrocyclic peptides. Microbiol Mol Biol Rev 70, 121-146.

Heather, L.C., Wang, X., West, J.A., and Griffin, J.L. (2013). A practical guide to metabolomic profiling as a discovery tool for human heart disease. J Mol Cell Cardiol 55, 2-11.

Horai, H., Arita, M., Kanaya, S., Nihei, Y., Ikeda, T., Suwa, K., Ojima, Y., Tanaka, K., Tanaka, S., Aoshima, K., et al. (2010). MassBank: a public repository for sharing mass spectral data for life sciences. J Mass Spectrom 45, 703-714.

Kopka, J., Schauer, N., Krueger, S., Birkemeyer, C., Usadel, B., Bergmuller, E., Dormann, P., Weckwerth, W., Gibon, Y., Stitt, M., et al. (2005). GMD@CSB.DB: the Golm Metabolome Database. Bioinformatics 21, 1635-1638.

Letzel, A.C., Pidot, S.J., and Hertweck, C. (2013). A genomic approach to the cryptic secondary metabolome of the anaerobic world. Nat Prod Rep 30, 392-428.

Losada, L., Ajayi, O., Frisvad, J.C., Yu, J., and Nierman, W.C. (2009). Effect of competition on the production and activity of secondary metabolites in Aspergillus species. Med Mycol 47 Suppl 1, S88-96.

Martin, F.P., Dumas, M.E., Wang, Y., Legido-Quigley, C., Yap, I.K., Tang, H., Zirah, S., Murphy, G.M., Cloarec, O., Lindon, J.C., et al. (2007). A top-down systems biology view of microbiome-mammalian metabolic interactions in a mouse model. Mol Syst Biol 3, 112.

Mootz, H.D., Schwarzer, D., and Marahiel, M.A. (2002). Ways of assembling complex natural products on modular nonribosomal peptide synthetases. Chembiochem 3, 490-504.

Newman, D.J., and Cragg, G.M. (2007). Natural products as sources of new drugs over the last 25 years. J Nat Prod 70, 461-477.

Niessen, W.M. (2003). Progress in liquid chromatography-mass spectrometry instrumentation and its impact on high-throughput screening. J Chromatogr A 1000, 413-436.

Nirmala, M.J., Samundeeswari, A., and Sankar, P.D. (2011). Natural plant resources in anti-cancer therapy-A review. Research in Plant Biology 1, 1-14.

Scalbert, A., Brennan, L., Fiehn, O., Hankemeier, T., Kristal, B.S., van Ommen, B., Pujos-Guillot, E., Verheij, E., Wishart, D., and Wopereis, S. (2009). Mass-spectrometry-based metabolomics: limitations and recommendations for future progress with particular focus on nutrition research. Metabolomics 5, 435-458.

Schumann, J., and Hertweck, C. (2006). Advances in cloning, functional analysis and heterologous expression of fungal polyketide synthase genes. J Biotechnol 124, 690-703.

Schwarzer, D., Finking, R., and Marahiel, M.A. (2003). Nonribosomal peptides: from genes to products. Nat Prod Rep 20, 275-287.

Schwarzer, D., Mootz, H.D., Linne, U., and Marahiel, M.A. (2002). Regeneration of misprimed nonribosomal peptide synthetases by type II thioesterases. Proc Natl Acad Sci U S A 99, 14083-14088.

Shaw-Reid, C.A., Kelleher, N.L., Losey, H.C., Gehring, A.M., Berg, C., and Walsh, C.T. (1999). Assembly line enzymology by multimodular nonribosomal peptide synthetases: the thioesterase domain of E. coli EntF catalyzes both elongation and cyclolactonization. Chemistry & biology 6, 385-400.

Shen, B. (2003). Polyketide biosynthesis beyond the type I, II and III polyketide synthase paradigms. Curr Opin Chem Biol 7, 285-295.

Staunton, J., and Weissman, K.J. (2001). Polyketide biosynthesis: a millennium review. Nat Prod Rep 18, 380-416.

Sumner, L.W., Amberg, A., Barrett, D., Beale, M.H., Beger, R., Daykin, C.A., Fan, T.W.-M., Fiehn, O., Goodacre, R., Griffin, J.L., et al. (2007). Proposed minimum reporting standards for chemical analysis. Metabolomics 3, 211-221.

Tolstikov, V.V., and Fiehn, O. (2002). Analysis of highly polar compounds of plant origin: combination of hydrophilic interaction chromatography and electrospray ion trap mass spectrometry. Anal Biochem 301, 298-307.

van den Berg, M.A. (2011). Impact of the Penicillium chrysogenum genome on industrial production of metabolites. Appl Microbiol Biotechnol 92, 45-53.

van den Berg, M.A.e.a. (2008). Genome sequencing and analysis of the filamentous fungus Penicillium chrysogenum. Nature biotechnology 26, 1161-1168.

van der Greef, J., Martin, S., Juhasz, P., Adourian, A., Plasterer, T., Verheij, E.R., and McBurney, R.N. (2007). The art and practice of systems biology in medicine: mapping patterns of relationships. J Proteome Res 6, 1540-1559.

von Wartburg, A., and Traber, R. (1988). Cyclosporins, fungal metabolites with immunosuppressive activities. Prog Med Chem 25, 1-33.

Wink, M. (2012). Medicinal plants: a source of anti-parasitic secondary metabolites. Molecules 17, 12771-12791.

Wishart, D.S., Knox, C., Guo, A.C., Eisner, R., Young, N., Gautam, B., Hau, D.D., Psychogios, N., Dong, E., Bouatra, S., et al. (2009). HMDB: a knowledgebase for the human metabolome. Nucleic Acids Res 37, D603-610.

Zerikly, M., and Challis, G.L. (2009). Strategies for the discovery of new natural products by genome mining. Chembiochem 10, 625-633.