Chapter 1

Introduction and scope of the thesis

Biologically active metabolites can be extracted from basically every living cell in nature and offer a plethora of medicines or structural templates for potential medicines. Therefore, the field of pharmaceutical biology is devoted to the extraction, purification, analysis and production of useful biologically active compounds. These compounds can be roughly divided into two groups: primary and secondary metabolites. Primary metabolites are often found in abundance and, at least the vast majority, are essential for survival of the organism. However, secondary metabolites are more species-specific, they do not have a role essential for growth, photosynthesis or other primary functions. Secondary metabolites consist of an extremely diverse group of chemical molecules with various functions. Although no official order exists for secondary metabolites, they can be divided roughly into three groups, namely: alkaloids, terpenoids and phenolics. Prominent alkaloids include morphine and caffeine; well-known phenolics are salicylic acid and lignin. Paclitaxel and cannabinoids are representatives of the terpenoid group.

Plant secondary metabolites are being used as medicines, flavors and fragrances and as recreational drugs. In order to be used by pharmaceutical industry the following steps are important: extraction of the compound, chemical characterization and large scale production.

Information about the localization, chemical structure and optimal time period for each specific product is crucial to facilitate extraction of these biologically active compounds. In practice this means performing pilot research based on traditional preparation methods or trial-and-error experiments. Traditional methods, commonly based on the historical reports from ayurveda and Chinese traditional medicine, can help in the initial screening processes and the extraction optimization. Despite this information, sometimes extractions have to be developed from scratch without prior knowledge. For use in traditional medicine and in pharmaceutical industrial, the extraction procedure is pivotal for attaining the compounds full potential. Co-extraction of other molecules can either negatively or positively influence the efficacy of the target compound.

After optimization of the extraction procedure and determination of the ideal harvest times and growth conditions, such metabolites need to be purified to allow their chemical characterization. Purified compounds are essential in order to establish efficacy of the single compound without the synergic effects of a mixture. In addition, pure compounds are essential for the elucidation of the chemical structure needed for organic synthesis of reference compounds or for the designing of a large-scale production process. The analytical procedure for such compounds is fully dictated by the chemical and physical characteristics of the specific compound under investigation. Traditional medicine usually adopts water or

alcohol based extraction procedures followed by chromatography (liquid and gas) for analysis. Although chromatography has certain limitations, it is often ideal for initial screening and purification. More recently, NMR is emerging as the first analytical choice. Beside the combination of NMR and chromatography, for specific structral information, NMR has certain benefits as it is non-destructive and provides robust structure-dependent signals for clasification of metabolites. For complex mixtures the clasification of metabolites depends heavily on statistical procedures, making proper noise filtering and data transformation essential (chapter 2). Yet large data sets can be prone to surprising correlations, such as the relation chocolate consumption, cognitive function, and Nobel laureates [1]. Some drawbacks of NMR are: limited sensitivity compared to other detection techniqes and expensive due to costly equipment and solvents. Therefore, hyphenated analytical techniques (gas and liquid chromatography) either coupled to photodiode array or mass spectrometry for the detection are still the first choice for the analysis of biologically active compounds due to their low price, high sensitivity or the possibility to separate compounds in a mixture.

After extraction, screening and purification of the active metabolite(s) of interest, the compound needs to be supplied in sufficient quantity for further clinical research. When the compound is found in abundance and its natural host is easily cultivated, this should not present a problem. However, often the natural abundance is low and therefore the production costs rise quickly. Alternatively, organic synthesis or biotechnological approaches can decrease production costs. Due to complexity of the chemical structure of most bioactive compounds, organic synthesis of these compounds can become a complex process [2] and suffering from low yields and/or high prices. Biotechnological approaches, however, are gaining more and more interest. Here it could be thought of traditional selection and breeding techniques, but also of the production of genetically altered hosts to increase the amount of produced compounds. Moreover, whole pathways can be transferred to classical laboratory organisms, such as baker's yeast, *Escherichia coli, Bacillus subtilis* and *Aspergillus spp*., to be able to produce the compound of interest by using and improving the natural enzymes involved in its assimilation [3-8]. This is known as metabolic engineering and has the potential to replace environmentally polluting organic synthesis. The natural host or genetic modifications of the natural host can offer a controllable production process with an optimized pathway.

At this moment metabolic engineering is a key player for industrial production processes. For instance, compounds isolated from *Stevia* (DSM; steviosides [9]) or *Artemisia* [10-12] are

produced by classical laboratory organisms using cheap organic compounds, like glucose obtained from inexpensive sources, such as molasses, starch or lignocellulose. Industrial biotechnology has set out to revolutionize conventional chemical manufacturing. Its benefit lies in the ability to modify cellular organisms. The preparations towards these approaches will be discussed in this thesis.

SCOPE OF THE THESIS:

The research described within this thesis is focused on the analysis of cannabinoid production patterns exhibited by selected strains from the medicinal *Cannabis* cultivator Bedrocan (Bedrocan B.V., Veendam). Furthermore, the enzymatic production of cannabigerolic acid is explored by analyzing the catalytic mechanism of a prenyltransferase. At the end the putative terpene based production host *Xanthophyllomyces dendrorhous* is tested for metabolic engineering purposes by the introduction of a terpenoid cyclase to serve as proof of principle and to obtain molecular strategies to genetically alter the host for non-native compound production, such as cannabinoids

Chapter 2 gives an overview of analytical strategies to analyze and identify metabolites from natural origin. The focus is especially on screening techniques to be able to detect as many molecules as possible, combined with *in vitro* screening data putatively responsible for the identification of biologically active molecules.

Chapter 3 gives an overview of cannabinoids and the research conducted over the past few years. It focuses especially on the chemical characteristics and biosynthesis.

Chapter 4 analyses the production pattern of cannabinoids by HPLC-UV and ^{1}H-NMR during the vegetative and flower cultivation periods. Moreover, the transcription of *cbda* and *thca* synthase mRNA is analyzed during the course of cultivation. This chapter highlights differences between the two *Cannabis sativa* strains, which are currently used and cultivated for medicinal purposes.

Chapter 5 describes a more focused approach for studying cannabinoid accumulation in specific plant organelles (trichomes) via laser dissection techniques.

Chapter 6 is intended to give insight into a novel catalytic mechanism of prenyltransferases and other terpene synthases. Using *in silico* simulation and predictions combined with site-directed mutagenesis studies, the catalytic mechanism of prenyltransferases is explored in detail with emphasis on cation stabilization involving the amino acid methionine.

Chapter 7 deals with the analysis of the residues lining the catalytic cavity of the prenyltransferase NphB. This enzyme has been shown to be able to produce cannabigerol after exposure to olivetol as substrate.

Chapter 8 analyses the host *Xanthophyllomyces dendrorhous* for its potential in metabolic engineering for terpenoid-based structures. As cannabinoids are combined molecules from a polyketide and terpenoid, this work can serve as preliminary research to produce cannabinoids and offers molecular strategies to fulfill this task.

"It has become appallingly obvious that our technology has exceeded our humanity."

– Albert Einstein