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CHAPTER 1

GENERAL INTRODUCTION

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Cannabis is an annual, dioecious plant, more rarely monoecious, native to central Asia. Cannabis most likely originates from Neolithic China and its preparations were known to the ancient Assyrians, Scythians, Chinese, Indians, and Persians. However the complete history of domestication is not known but the first known evidence of the use of cannabis as a medicine was found in China 500 years ago (Hanuš and Mechoulam 2005). Preparations of cannabis have been used by man for their euphoric effects for over 4000 years (Uhl 2006). More than 700 varieties of Cannabis have been reported, however, the species and chemotaxonomic classification of this plant is quite controversial. Schultes et al. (1974) described three putative varieties of cannabis, *Cannabis sativa* L. (tall branched plants for fiber, seed and psychotropic use), *Cannabis indica* Lam. (short, broad leaflets plants utilized to produce hashish), *Cannabis ruderalis* Jan. (short, unbranched road side plants). More recently, de Meijer developed a classification system of cannabis based on the cannabinoid contents (de Meijer et al. 2003). For forensic and legislative purposes, the cannabis types are classified into the drug types and the fiber types (Hazekamp and Fischedick 2012). This classification is based on the contents of the psychoactive cannabinoid Tetrahydrocannabinol (THC). The ratio (THC + CBN)/CBD is determined for identifying the phenotypes of cannabis plants (Fetterman et al. 1971). If the ratio found is greater than 1, the cannabis variety is classified as drug type; if it is less than 1, it is a fiber type. More simply, cannabis strains cultivated for fiber and/or seed purposes (with low content of THC) are referred as fiber type, while the strains cultivated for medicinal or recreational use (with high content of THC) are recognized as drug type (Hillig and Mahlberg 2004, Hossein and Isaac 2007).

The basic material of all the cannabis products is the plant *Cannabis sativa* L. (Hazekamp 2007). Therefore, in literature more often, the variety *C. sativa* L. refers to cannabis. Likewise, in this thesis *C. sativa* will be used to denote

cannabis and vice versa. *Cannabis sativa* encompass both drug and nondrug varieties. Nondrug varieties are called hemp and drug varieties are referred to as marijuana (Hossein and Isaac 2007). The enormous number of products derived from *C. sativa* have greatly increased the attention for the chemistry and pharmacology of the plant. The demand for hemp made products is increasing in the global market, including fibers hemp leaves, hemp seed derivatives, oil, flour, beverages (beer, lemonade and liqueur) and cosmetic products (Lachenmeier et al. 2004). Hemp is becoming the centre of attention for sustainable economic development in USA (Tun 2005). The medicinal applications of marijuana are also intensively investigated. A large number of clinical reports favor the use of marijuana as an effective remedy against e.g. central thalamic pain, dystonia, familial Mediterranean fever, multiple sclerosis, chronic pain, depression, anxiety, migraine and sleeping difficulties (Ware et al. 2005). *Cannabis sativa* is a rich source of a variety of compounds; more than 500 chemical compounds have been identified in this plant, including cannabinoids, terpenoids and flavonoids. The synergism between these compounds might play a role in the therapeutic potential of cannabis (Stott and Guy 2004).

The term "cannabinoids" represents a group of C1, C3 and C5 side chains terpenophenolic compounds only found until now in *C. sativa* (Cannabaceae). The highest concentration of cannabinoids is present in resinous form in the buds and flowering tops of the female plants (Mechoulam and Goani 1967). After the purification and structure elucidation of Δ^9-Tetrahydrocannabinol (Δ^9-THC) (Gaoni and Mechoulam 1964), Cannabidiol (CBD), Cannabiniol (CBN), and Δ^8-Tetrahydrocannabinol (Δ^8-THC) (El-Sohly 2006) were extensively studied and found to possess a variety of potentially useful pharmacological activities in addition to the psychotropic effects for which cannabis is well known (Monique and Stephen 1976). Cannabinoids, the active compounds in *C.*

sativa have many distinct therapeutic properties (Sirikantaramas et al. 2007). The analgesic, anti-emetic, anti-oxidative, neuroprotective and anti-inflammatory properties could be helpful in the treatment of many neurological disorders (Carter and Weydt 2002). Physiological actions as anticonvulsant, antidepressant, hypotensive, bronchodilation and lowering of intraocular pressure have led to a number of investigations on the possible development of useful medicines from the naturally occurring and synthetic cannabinoids. The Δ^9-THC is the psychoactive component of the cannabis plant (Scotter et al. 2010), while the major non psychoactive constituents include CBD, CBN, cannabigerol (CBG) and cannabichromene (CBC) (Gaoni and Mechoulam 1966). In the plant these compounds occur in their respective acidic form. i.e. having a carboxylic acid group. The acidic cannabinoids, which are non–psychotropic, undergo decarboxylation upon heat treatment (e.g. smoking) to yield the physchotropic cannabinoids.

The Δ^9-THC is known to be responsible for the main psychotropic effects of the cannabis made preparations (Ashton 2001). It was first identified in 1964 by Mechoulam and Gaoni (Gaoni and Mechoulam 1964). Sometimes, Δ^9-THC is used as a marker in evaluating the drug intensity of various cannabis based preparations (hashish, marijuana, hash oil) (Gambaro et al. 2002). The Δ^9-THC acts on the central nervous system (CNS) and caused euphoria, relaxation, tachycardia and alteration in blood pressure; hallucinations also appear at high doses (Pellegrini et al. 2005). It has been used in a number of disease states (e.g. pain, anxiety, asthma, glaucoma, and hypertension) (Abbott et al. 1977). Δ^9-THC mediates many of these effects by acting on the cannabinoid receptor type 1 (CB_1) (Tseng and Craft 2004, Varvel et al. 2005). The synthetic Δ^9-THC is being prescribed with the brand name “Marinol”. In 1986, the U.S food and drug administration (FDA) approved Marinol for the treatment of nausea and vomiting caused by cancer therapy. Marinol has also been used as an antiemetic

drug to treat loss of appetite and weight loss in patients infected with HIV (the virus causes AIDS). A U.K based company (GW Pharmaceuticals) sells the drug named Sativex, which contains the two best known cannabinoids, Δ^9-THC and CBD. Sativex has been approved in Canada, New Zealand and eight European countries. This drug is effective against muscle spasms associated with multiple sclerosis (MS), cancer and neuropathic pain.

After 5 decades of research, a lot is known about the chemistry and pharmacology of Δ^9-THC. Surely, Δ^9-THC has been approved by FDA and is being marketed to the pharmacies of U.S.A and Canada. But still the poor water solubility of Δ^9-THC is an issue which needs to be addressed. Δ^9-THC is a lipophilic compound and only slightly soluble in water. The human body contains high lipid contents, which are not only the body fats but also present in brain and cell membranes. Lipid-soluble drugs leave the blood rapidly and tend to accumulate in the fatty tissues (Hollister 1998). Likewise, Δ^9-THC binds strongly to plasma protein and other fatty tissues, which prolongs its release from the body (Paton 1975). The intravenously administered Δ^9-THC to a human can persists longer than three days in the plasma and its metabolites can be detected in the urine or feces even for 8 days (Kanter and Hollister 1977, Lemberger et al. 1970). Although, there is no report of continuing effects of the drug, the long persistence of Δ^9-THC in the body and the slow clearance of its metabolites might cause side effects. So, there is a need to structurally transform the compound to increase its polarity and its rapid release from the body.

Biotransformations are the chemical reactions carried out by cells, organs and enzymes. These reactions are used to structurally modify the compounds by exploring the unique properties of biocatalysts (Giri et al. 2001). Δ^9-THC was transformed into a number of new derivatives by using mammalian enzyme systems, leading to a better understanding of their pharmacodynamics and

pharmacokinetics. Mammalian transformations were useful to reveal the various metabolic pathways of Δ^9-THC in different mammalian species, but for the industrial scale production of the potential compounds by means of mammalian cell culture or tissue culture is too expensive to develop cannabinoid based drugs. Microorganisms may perform similar biotransformations as mammalians and could be suitable source for large scale production of new cannabinoids. The biotransformation potential of microorganisms and their enzymes for the production of new modified compounds is well acknowledged. The attractive characteristics of microorganisms make them favorable for biotransformation studies because of reduction in the number of animals used, ease of setup and manipulation (can easily be scaled up), and maintenance of stock cultures is simple and cost effective. Plants are able to produce a number of different chemicals which cannot easily be produced by synthetic means. Plant cell cultures are used to produce a variety of secondary metabolites including phenolics, steroids, alkaloids, coumarins and terpenoids. Plant enzymes act as unique biocatalysts and can successfully transform exogenous substrate to novel substances. Microorganisms and plant cell cultures have successfully produced new cannabinoids, which are structurally similar to the compounds obtained from mammalian transformations (This thesis). The only need is to scale up the production of these metabolites for further bioassays, clinical studies and potential commercial uses.

The zebrafish embryo is emerging as a prominent model of developmental biology, for disease studies and for drug discovery. A number of unique characteristics of the zebrafish embryo makes it an attractive model: small size, ease of maintenance, rapid development, large number of offspring, small amount of compound required for testing, and its optical transparency (Dahm and Geisler 2006). The embryos hatch approximately 2-3 dpf (Days post fertilization) and organogenesis of major organs is completed at 5 dpf

(Rubinstein 2003). The transparency of embryos and young larvae provide insight in the organ formation and their functions in developmental processes (Schwerte and Fritsche 2003). This optical transparency and access to all the developmental stages give an opportunity to study developing pathologies at different stages (Lieschke and Currie 2007). Genome sequencing has revealed a great deal of homology between the zebrafish and other vertebrates (including humans) (Schwerte and Fritsche 2003). A number of sophisticated mutagenesis and screening approaches have been developed in zebrafish embryos and made it a model of choice for the study of a wide variety of human diseases. Different protocols and automated imaging systems have been established for the behavioral analyses of zebrafish larvae. These systems produce a large set of data and facilitate high-throughput genetic, pharmacological and environmental screening (Creton 2009). A range of simple sensorimotor responses appear in the early developmental stages of the zebrafish larvae. These features represent the brain development and are useful for behavioral investigations (Souza and Tropepe 2011).

The zebrafish larvae are not only useful for high throughput analyses or acute toxicity but also helpful to understand the mechanisms of toxicity and possible adverse and long term effects of hazardous chemicals. The effect of added chemical entities finally leads to an alteration in gene transcription and protein expression, which ultimately affects the metabolic profile of the organism. Metabolites are the final product of gene expression and metabolomic profiling leads to the understanding of possible important events taking place in a cell, tissue or organism (Hayashi et al. 2011). A qualitative and quantitative measurement of all the metabolites present in a system at a particular time is called Metabolomics. Metabolomics, together with transcriptomics and proteomics, reflects the condition of the system and may show e.g. the effect of a medicine or toxin on the organism (Scholz et al. 2008). Metabolic

fingerprinting has been successfully introduced in zebrafish larvae to predict different embryonic stages (Hayashi et al. 2011, Hayashi et al. 2009). A multi-analytical approach (including 1H NMR, GC/MS and LC/MS) was also used to study the biochemical profile of livers of male and female zebrafish (Ong et al. 2009).

The CB1 (Lam et al. 2006) and CB2 (Rodriguez-Martin et al. 2007) receptors have been identified in zebrafish. A high level of sequence conservation of the CB_1 receptor has been shown for zebrafish and mammals (Lam et al. 2006). Involvement of the CB_1 receptor is also reported in the hatching process of zebrafish embryo (Migliarini and Carnevali 2009). The cannabinoid receptors in zebrafish embryos can help in studies to gain further insight in the pharmacology of cannabinoids and it might also be helpful to resolve some unclear features of the cannabinoids mode of action, like the phenomenon of tolerance and dependence caused by cannabinoid based drugs (Rodriguez-Martin et al. 2007).

Aim of the thesis

The aim of the present study was:

- To investigate the potential of bacterial strains and plant cell cultures to produce polar derivatives of Δ^9-THC.

- To examine the possibilities of using the zebrafish embryo to screen for cannabis receptor affecting compounds.

For the biotransformation, a library of microorganisms able to survive on hydrocarbons was available for screening. Methods for the analysis of cannabinoids and their metabolites and for their isolation and structure elucidation by spectroscopic methods were required for this study.

For the zebrafish screening the developmental, behavioral and metabolic effects of known cannabinoids in zebrafish embryos by using whole mount staining, visual motor response test needed to be explored. Furthermore a novel systems biology approach, using metabolomics was developed to study the effect of the cannabinoids.

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