Chemistry and biological activity of Tetrahydrocannabinol and its derivatives

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1 Abstract

Cannabinoids and in particular the main psychoactive Δ9-THC are promising substances in the drug development process and of high importance in biomedicine and pharmacy. This review gives an overview about chemical properties of Δ9-THC, its synthesis in industrial scale and also the synthesis of important metabolites. The biosynthesis of cannabinoids in *Cannabis sativa* is extensively described, in addition strategies for optimization of this plant with respect to their employment in medicine are discussed. The metabolism of Δ9-THC in humans is shown and and based on this facts analytical procedures for cannabinoids and their metabolites in human forensic samples as well as in *Cannabis sativa* will be discussed. Furthermore some aspects are elucidated regarding medicinal indications of Δ9-THC and its ways of administration. Moreover some synthetic cannabinoids and their importance in research and medicine is delineated.

2 Chemistry

2.1 Nomenclature

Natural cannabinoids are terpenophenolic compounds which are only biosynthesised in *Cannabis sativa* L., Cannabaceae. For these compounds five different systems of nomenclature are available, well described by Shulgin [1] and furthermore by ElSohly [2]. Two of these systems are mainly employed for the description of tetrahydrocannabinol in publications – the dibenzopyrane numbering system (Figure 1.1) and the terpene numbering system (Figure 1.2), based on *p*-cymene. Because of historic and geographical reasons the missing standardisation is not uniform and main reason for ongoing confusion in literature leads to discussion in scientific literature regarding the numbering and its order. As an example, the use of the terpene numbering system gives the name Δ1-tetrahydrocannabinol, in contrast using the dibenzopyrane numbering system leads to the name Δ9-Tetrahydrocannabinol for the same compound. The dibenzopyrane numbering system, which stands in agreement with IUPAC rules, is commonly used in North America whereas the terpene numbering system - following the biochemical nature of these compounds - was originally developed in Europe [3]. According to IUPAC rules the dibenzopyrane system is used despite the fact that the dibenzopyrane system has a general disadvantage because of a complete change in numbering after loss of the terpenoid ring as it can be found in many cannabinoids.

Figure 1

The chemical name of Δ9-THC according to the dibenzopyrane numbering system is 3-pentyl-6,6,9-trimethyl-6a,7,8,10a-tetrahydro-6*H*-dibenzo[*b,d*]pyran-1-ol as depicted in Fig. 1.1.
Alternatively Δ9-tetrahydrocannbinol or only tetrahydrocannabinol is frequently used in the scientific community. When using the short name tetrahydrocannabinol or just THC it implies always the stereochemistry of Δ9-isomer.
On the market are two drugs under the trade names of Dronabinol®, which is the generic name of *trans*-Δ9-THC, and Marinol®, which is a medicine containing synthetic Dronabinol in sesame oil for oral intake, distributed by Unimed Pharmaceuticals Inc.

2.2 Chemical and physical properties of Δ9-THC

Δ9-THC (Fig. 2.1) is the only major psychoactive constituent in *C. sativa*. It is a pale yellow resinous oil and sticky at room temperature. Δ9-THC is a lipophilic and in water poor soluble compound (3 µg mL^{-1}) with a bitter taste but without smell. Furthermore it is sensitive to light and air [4-6]. Some more physical and chemical data of Δ9-THC are listed in Table 1. Because of its two chiral centres at C-6a and C-10a four stereo isomers are known, but only (-)-*trans*-Δ9-THC is found in the *Cannabis* plant [7]. The absolute configuration of the natural product was determined as (*6aR,10aR*) [8]. Depending on the position of the double bond in the terpenoid ring six isomers are possible whereof the Δ9-isomer and the Δ8-isomer are most important. Conformational studies of Δ9-THC by using NMR-techniques were done by Kriwacki and Makryiannis [9]. The authors found out that the arrangement of terpenoid ring and the pyrane ring of this compound is similar to the halfway opened wings of a butterfly. An excellent review by Mechoulam et. al. has been published providing more information on this topic and discussing extensively the stereochemistry of cannabinoids and Δ9-THC with special focus on the structure-activity-relations [10].

Table 1

It must be noted that in *C. sativa* Δ9-THC is not present, but the tetrahydrocannbinolic acid (THCA) is almost exclusively found. Two kinds of THCA are known. The first one has its carboxylic function at position C-2 and is named 2-carboxy-Δ9-THC or THCA-A (Fig. 2.2), the second one with a carboxylic function at position C-4 is named 4-carboxy-Δ9-THC or THCA-B (Fig. 2.3).
THCA shows no psychotropic effects, but heating (e. g. by smoking of *Cannabis*) leads to decarboxylation which provides the active substance Δ9-THC. Δ9-THC is naturally accompanied by its homologous compounds containing a propyl side chain (e.g. Tetrahydrocannabivarin, THCV, THC-C_3, Fig. 2.4) or a butyl side chain (THC-C_4, Fig. 2.5).

Further natural Cannabinoids

Seventy cannabinoids from *C. sativa* were known until 2005 [2]. Mostly they appear in low quantities, but some of them shall be

mentioned in the following overview – especially because of their functions in the biosynthesis of Δ9-THC and their use in medicinal applications.

2.3.1 Cannabigerol (CBG)

Cannabigerol (CBG, Fig. 2.6) was historically the first identified cannabinoid [11]. It can be comprehended as a molecule of olivetol which is enhanced with 2,5-dimethylhepta-2,5-diene. In plants its acidic form cannabigerolic acid (CBGA, Fig. 2.7) and also the acid forms of the other cannabinoids are prevailing. CBGA is the first cannabinoidic precursor in the biosynthesis of Δ9-THC as discussed in subheader 3. Although the n-pentyl side chain is predominant in natural cannabinoids also cannabigerol with propyl side chains (cannabigerovarin, CBGV, Fig. 2.8) are present.

2.3.2 Cannabidiol (CBD)

The IUPAC name of cannabidiol is 2-[(*1S,6R*)-3-methyl-6-prop-1-en-2-yl-1-cyclohex-2-enyl]-5-pentyl-benzene-1,3-diol. Cannabidiol (CBD, Fig. 2.9) respectively its acidic form cannabidiolic acid (CBDA, Fig. 2.10) is the second major cannabinoid in *C. sativa*. besides Δ9-THC. As already mentioned for Δ9-THC variations in the length of the side chain also possible for CBD. Important in this context are the propyl side chain substituted CBD, named Cannabidivarin (CBDV, Fig. 2.11) and CBD-C_4 (Fig. 2.12), the homologous compound with a butyl side chain. Related to the synthesis starting from CBD to Δ9-THC as described in subheader 4.1 it was accepted that CBDA serves as a precursor for THCA in the biosynthesis. Recent publications indicate that CBDA and THCA are formed from the same precursor Cannabigerolic acid (CBGA) and it is unlikely that the biosynthesis of THCA from CBDA takes place in *C. sativa*.

2.3.3 Δ8-Tetrahydrocannabinol (Δ8-THC)

This compound and its related acidic form, Δ8-Tetrahydrocannabinolic acid (Δ8-THCA, Fig. 2.13) are structural isomers of Δ9-THC. Although it is the thermodynamically stable form of THC, Δ8-THC (Fig. 2.14) contributes with approximately only one

percent to the total content of THC in *C. sativa*. In the synthetic production process Δ8-THC is formed in significant higher quantitites than in plants.

2.3.4 Cannabichromene (CBC)

Among THCA and CBDA Cannabichromene (CBC, Fig. 2.15) respectively the acidic form named cannabichromenic acid (CBCA, Fig. 2.16) is formed from their common precursor CBGA. Beside CBC its homologous compound Cannabiverol (CBCV, Fig. 2.17) with a propyl side chain is also present in plants.

2.3.5 Cannabinodiol (CBND) and Cannabinol (CBN)

Cannabinidiol (CBND, Fig. 2.18) and Cannabinol (Fig. 2.19) are oxidation products of CBD and Δ9-THC formed by aromatisation of the terpenoid ring. For the dehydrogenation of THC a radical mechanism including polyhydroxylated intermediates is suggested [12, 13]. CBN is not a sole oxidation product of Δ9-THC. Own studies about stability of Δ9-THC at THC-Pharm GmbH have shown that merely about 15% of lost Δ9-THC are recovered as CBN.

Figure 2

3. Biosynthesis of cannabinoids

The biosynthesis of cannabinoids can only be found in *C. sativa*. These cannabinoids are praised for their medical and psychoactive properties. In addition, the plant material is used for fiber, oil and food production [14]. For these applications it is important to gain knowledge of the cannabinoid biosynthetic pathway. As an example, fiber production is not allowed if the plant contains more than 0.2% (of dry weight) THC. Higher THC content is illegal in most Western countries and cultivation is strictly regulated by authorities. Interestingly the content of other cannabinoids is of less importance because no psychoactive activity is claimed for them. Furthermore, for forensic purposes the information may be used to discriminate the plants by genotype, which is correlated to the chemotype (see 3.1.2), in early phase of their development. This may help both the cultivator and legal forces. Here the cultivation of illegal plants may be found

and controlled by both of them. For the cultivator, to exclude illegally planted plants and for the police to control illegal activities by the cultivators or criminals. Moreover, the information can be used by pharmaceutical companies and scientists. Here it can be used for the studies on controlled production of specific cannabinoids that are of interest in medicine. For instance, THC has been investigated to temper the symptoms of multiple sclerosis [15], but also CBG and CBD can play a role in medicine. Both CBD and CBG are related to analgesic and anti-inflammatory effects [16, 17].
In this paragraph latest developments and recent publications of the biosynthesis of Δ9-THC and related cannabinoids as precursors are discussed. Special points of interests are genetic aspects, enzyme regulation and environmental factors which have an influence on the cannabinoid content in the plant. Because of new and innovative developments in the biotechnological field we will give a short outlook in new strategies for cannabinoids production in plant cell cultures and hetrologeous organisms.

3.1 Biochemistry and Biosynthesis

The biosynthesis of major cannabinoids in *C. sativa* is located in the glandular trachoma, which are located on leaves and flowers. Three known resin producing glandular trachoma are known, the bulbous glands, the capitate sessile and the capitate stalked trichoma. It was described that the latter contain most cannabinoids [18]. The capitate stalked becomes abundant on the bracts when the plant ages and transfer into the flowering period. The capitate sessile trichoma show highest densities during the vegetative growth [19, 20].
As depicted in Fig. 3, in glandular trichoma the cannabinoids are produced in the cells but accumulate in the secretory sac of the glandular trichomes dissolved in the essential oil [19-23]. Here, Δ9-THC was found to accumulate in the cell wall, the fibrillar matrix and the surface feature of vesicles in the secretory cavity, the subcutilar wall, and the cuticula of glandular trichomes [21].

Figure 3

As mentioned before, the cannabinoids represent a unique group of secondary metabolites called terpenophenolics, which means that they are composed of a terpenoid and a phenolic moiety. The pathway of terpenoid production is already reviewed exhaustingly [24-27]. The

phenolic unit of cannabinoids is thought to be produced via the polyketide pathway [28-30]. Both, the polyketide and terpenoid pathways merge to the cannabinoid pathway and this combination leads to the final biosynthesis of the typical cannabinoid skeleton. Here we will discus the different aspects of the cannabinoid pathway for most already found cannabinoids, like cannabigerolic acid (CBGA), tetrahydrocannabinolic acid (THCA), Cannabidiolic acid (CBDA), and cannabichromenic acid (CBCA), as introduced before. For convenience the abbreviations of the acidic form will be used through this paragraph because they occur as genuine compounds in the biosynthesis. Under plant physiological conditions the decarboxylated products will be absent or present only in small amounts.

The late cannabinoid pathway starts with the alkylation of olivetolic acid (Fig. 4.2) as polyketide by geranyl diphosphate (Fig 4.1) as terpenoid unit. Terpenoids can be found in all organisms, and in plants two terpenoid pathways are known, the so called mevalonate (MEV) and non-mevalonate (DXP) pathway as described by Eisenrich, Lichtenthaler and Rohdich [25, 26, 31, 32]. The mevalonate pathway is located in the cytoplasm of the plant cells [32], whereas the DXP pathway as major pathway is located in the plastids of the plant cells [31] and delivers geranyl diphosphate as one important precursor in the biosynthesis.

The polyketide pathway for olivetolic acid is not yet fully elucidated. It is assumed that a polyketide III synthase will either couple three malonyl-CoA units with one hexanoyl-CoA unit [28], or catalyze binding of one acetyl-CoA with four malonyl-CoA units [30] to biosynthesize olivetolic acid [28-30, 33, 34]. Olivetolic acid as precursor for Δ9-THC contains a pentyl chain in position C3 at its phenolic system, but shorter chain lengths have also been observed in cannabinoids [35]. These differences in chain length support the hypothesis of production by a polyketide, as it is a known feature of these enzymes [36]. It was recently described that crude plant cell extracts from *C. sativa* are able to convert polyketide precursors into olivetol [28]; however here no olivetolic acid was detected. On the contrary Fellermeier et al. [34] showed that only olivetolic acid and not olivetol could serve in the enzymatic prenylation with GPP or NPP. An older article described that both the olivetol as olivetolic acid can be incorporated. Here the incorporation of radioactive labeled olivetol has been detected in very low and olivetolic acid in high amounts. These reactions were performed In Planta, whereas the previous reactions were performed in vitro [37]. Until know it still

remains unclear which structure, olivetol or olivetolic acid, is really preferred. Horper [38] and later Raharjo [28] suggested that the aggregation of the enzymes could prevent the decarboxylation of olivetolic acid. This explanation suggests that the enzymes are either combined or closely located to each other so that the olivetolic acid is placed directly into the site responsible for prenylation. This hypothesis has still to be proven, but supports the fact that olivetolic acid can not be found in *Cannabis* extracts [37].
Until recently no enzymes able to produce olivetol-like compound were isolated. In an article by Funa et al., 2006, polyketide III enzymes were responsible for the formation of phenolic lipid compound [36], a natural product group that olivetol belongs to. Although the biosynthesized compounds contained a longer chain, which increased during time, the study support the hypothesis of olivetolic acid production by a polyketide III synthase. Further studies on the genetic and protein level are essential to elucidate the mode of mechanism by which olivetolic acid is formed in *C. sativa*.

The precursor of the major cannabinoids is proven to be cannabigerolic acid (CBGA, Fig 4.3) [34, 37]. The formation of this compound is catalyzed by an enzyme from the group geranyltransferase [30, 34]. This enzyme was studied in crude extracts made from young expanding leafs, were it exhibited activity only with olivetolic acid as the substrate. Despite the fact that no sequence has been published yet, the enzyme was designated geranylpyrophosphate: olivetolate geranyltransferase (GOT). Recently [39] the structure and characterization of a geranyltransferase, named orf-2 and originating from *Streptomyces* CL109, was reported. The authors claimed that the enzyme is able to geranylate both olivetol and olivetolic acid and thus it may be highly similar to the CBGA synthase. Although the authors made this firm statement, they based it on the results obtained by thin layer chromatography. Thus for confirmation of this activity more precise analytical techniques, like LC-MS or NMR, must be performed for structure elucidation of the product produced. Although we have more information about GOT than the polyketide synthase (see Table 2), it remains unsure what is the mechanism of activity. This means more studies must be performed to obtain the gene sequence.

Table 2

The last enzymatic step of the cannabinoid pathway is the production of THCA (Fig. 4.5), CBDA (Fig 4.4) or CBCA (Fig. 4.6). The

compounds are produced by three different enzymes. The first enzyme produces the major psychoactive compound of Cannabis, the THCA [23, 40] the second and third are responsible for the production of CBDA [41] and CBCA [42] respectively. All of these enzymes belong to the enzyme group oxidoreductases [40-43], which means they are able to use an electron donor for the transfer of an electron to an acceptor. From these enzymes only the THCA and the CBDA synthase gene sequence have been elucidated. Their product also represents the highest constituent in most *C. sativa* strains.

The enzyme responsible for THCA formation is fully characterized and cloned into several hetrologeous organisms. When cloned in a hosts organisms, the highest activity was mostly seen in the media. Here the only exception was the introduction of the gene in hairy root cultures made from tobacco [44]. Studies performed on the enzyme sequence indicated that it contains a signal sequence up stream of the actual enzyme. This was found to be 28 amino acids (84 bp) long, suggesting that the enzyme, under native conditions, is localized to another place than it is produced. Later studies proved that the enzyme is localized in the storage cavity of the glandular trichomes [23]. In the first publication it was determined that no cofactor is used by the enzyme [43], but this research was performed with purified protein from the *C. sativa* extract. Later studies indicated that a Flavin adenine dinucleotide (FAD) cofactor was covalently bound to the enzyme. This was later confirmed by nucleotide sequence analysis in silico, revealing the binding motive for the FAD co-factor.

The CBDA synthase is though to be an allozyme of THCA synthase and shows 87.9 % identity on a nucleotide sequence level. Although the sequence of this gene is known [45], there are no report of studies where they produced and characterized it. All information gained over the enzyme is obtained by purified protein from *C. sativa* extracts [41]. Although not tested yet, the deposited sequence shows the same conserved FAD binding motive as found and proven for THCA synthase. Because the CBDA synthase carries the same signal sequence as the THCA synthase it may be suggested that the CBDA is localized in the same place as the THCA synthase.

For CBCA synthase hardly any information is published. The enzyme is characterized after it was purified from *C. sativa* extracts and until this moment no sequence has been deposited. After purification of the protein it was found to be a homodimeric enzyme, meaning that enzyme is formed by two identical domains. This was observed after the purification when the enzyme had a molecular weight of 136 kDa, and after denatured electrophoresis it had a molecular weight of ~71 kDa. Furthermore, the CBCA synthase has shown to bear higher affinity for the CBGA (1717 $M^{-1}S^{-1}$) than THCA- and CBDA synthase

(respectively 1382 $M^{-1}S^{-1}$and 1492 $M^{-1}S^{-1}$), which is probably due to its homodimeric nature [42].

From the biosynthetic route a lot of knowledge is gathered trough the years. Up to now only one enzyme is reasonable characterized, but much information is already gained trough crude extract activity studies. This information has already proven to be a solid basis for genetic testing and will be useful for further investigations of the biosynthetic route. Although it must be stated that high polymorphism is detected in the genes [46] and high genetic diversity found within *C. sativa* can still give unexpected results in other investigations. The information gained from the research reported above is already used frequently in the breeding and detection of certain chemotypes and the development of new ones, as we will see further on in this paragraph.

Figure 4

3.2 Genetics of *Cannabis sativa*:

The majority of *C. sativa* strains exists as a dioeciously (separate sexes) plant species and is wind pollinated. Under normal condition it is an annual herb, although longer living *C. sativa* have been observed [47, 48]. Some *Cannabis* strains appear as monoecious (contains both male as female parts) cultivars, such as the Ukrainian cultivar USO31 [49], or as hermaphrodites. Most of these cultivars are not seen in nature. It is estimated that only 6% of the flowering plants are dioecious and generally they are seen as the most evolved species within the plant kingdom [50, 51]. The *C. sativa* genome is normally a diploid one and contains 10 chromosome pairs (2n=20).Here, eighteen are autosomal- and two are sex-linked chromosome. The genome was measured in both female (XX) as well as male plants (XY). In contrary to animals, the male genome was found to be bigger by 47 Mbp [52, 53]. It must be stated that dioecious plants are able to change sex during their development. This ability is mostly used as a strategy for survival, however it can be chemically induced. Within the *C. sativa* species lots of phenotypes are known. Generally the *C. sativa* plant are believed to be a monotypic specie [49] called *Cannabis sativa* L with further divisions in subspecies. However, Hillig [48] showed, by allozyme analysis in combination with morphological traits, that a separation may be made between *C. sativa* L. and the *C. indica* Lam. He also suggested a putative third one named *C. ruderalis* Janisch. The polytypic species within *C. sativa* was already suggested several years ago when the plants were

determined only by their phenotypic traits or drug potential properties [48]. Until now there is still discussion about whether or not the *C. sativa* species are monotypic or polytypic, but in most literature they are referent as *C. sativa* with further division in the subspecies *indica* or *ruderalis*.

C. sativa is mostly divided in three major chemotypes. The chemotypes boundaries are set by the ratio CBD:THC and are calculated by the percentage of the dry weight. These three chemotypes consist of the 'fiber'-type (CBD>THC), the 'intermediate'-type (CBD≈THC) and the 'drug'-type (CBD<THC). The chemotypes have been recently shown to be dependent either on one locus on the chromosome, or two closely linked loci [49], but the former theory is the most likely one [54-56]. The locus is called B locus and until now it is proven to consist of at least two alleles, namely B_t and B_d. There is also an indication for a third allele. This one was named B_0 and seems to be responsible for a CBGA dominant chemotype [56]. The alleles, B_t and B_d, show co-dominance and the B_0 allele is recessive or an inactive B_d allele. The B_0 is believed to be an inactive B_d allele because it can be indicated by molecular markers specific for the CBDA gene (B_d allele). The evidence for these alleles was gained by breeding with chemotypic and molecular analysis [49]. In crossings made with fiber type and drug type, intermediate chemotype was obtained as offspring . Intercrosses of these F1 plants gave a representative Mendelian ratio (1:2:1) of chemotypes. This Mendelian ratio suggests that one locus is responsible for the chemotypes. Furthermore, Pacifico et al [49] proved, with the help of multiplex PCR, a 100% identification of specific chemotype (from the three accepted chemotypes) could be made. This multiplex PCR was performed with three primers, one of which was designed to anneal with both the THCA synthase and the CBDA synthase gene, while the other two were specific for one of both. The results showed that the intermediate chemotype was heterozygote and thus contained both the CBDA synthase and the THCA synthase genes. The drug and the fiber type were shown to be homogeneous for the THCA synthase and the CBDA synthase genes respectively. Although the genes are not themselves detected their products are. For instance, in the fiber-type group that is shown to be homogenous for the B_d allele is still producing low amounts of THCA. It is thus still possible that the homogeneous type is still carrying the THCA synthase gene however it is not detected due to the polymorphisms within the gene, as shown by Kojoma et. al. [46].

Recently it has been suggested that there are two more chemotypes. The first (chemotype 4) has a high content of CBGA (B_0 allele) and

the second one is a strain totally lacking cannabinoids [49]. These strains are of interest because they can serve as good and save strain for the production of fiber.

3.3 Environmental factors

Cannabis seems to react on several environmental influences. The most known are hydration, soil nutrients, wounding, competition and UV-B radiation. Proper use of these environmental influences can increase the Glandular density and the cannabinoid content. Environmental factors have also shown to induce sex change in *C. sativa*. More over when some chemotypes are grown in a different environment their cannabinoid content seem to be changed. With genetic analysis it must be possible to determine if this strain was indeed a fiber strain or if it was an intermediate strain which was suppressed for its cannabinoid content due to the environment of cultivation. Some of the major environmental factors influencing the cannabinoid content are described below. It must be stated that environmental stress also affects the growth of the plant.

3.3.1 Dehydration

In times of less accessibility of water, the plants seem to increase the cannabinoid content. It is suggested that the plant will cover itself with the oily cannabinoids to prevent water evaporation. For instance Sharma (1975) found increased glandular trichome densities in the leaves of Cannabis grown under dry circumstances [57].

3.3.2 Nutrients in soil

It is clear that the nutrients in soil are important for plant development and that a good nutrient supply within the soil gives healthy plants. However, for the most optimal conditions of soil still no profound research results have been published.

3.3.3 Light

Light has a major influence on plants because and for *Cannabis* plants it is mostly important for growth and flowering. Long daylight induces strong vegetative growth and shorter daylight leads to

flowering of the plants. Furthermore, it has been shown by Lydon et al. that the level of THC increase is linear with the increase of UV-B dose [58-60].

3.4 Growing of *Cannabis sativa* and optimization of THC yield

3.4.1 Cultivation of Cannabis

C. sativa is cultivated for several purposes. Actually, the main legal purpose is the production of hemp fibers and pulp. From these materials paper, clothes and ropes are made [14] and several western countries already legalized the cultivation of *C. sativa* for these purposes. In research the drug type of *C. sativa* is also cultivated, however only for the investigation and determination of forensic studies for chemotype separation. The growth for medicinal purposes is hardly performed. In the Netherlands *C. sativa* is cultivated for medicinal purposes under strictly controlled regulations by the company Bedrocan b.v. In this chapter we discuss basic aspects of the cultivation of C. sativa and the optimization of THC content in the plant.

3.4.2 Optimisation of THC yield

The optimization of THC yield is mostly performed through breeding programs. Because of the illegality of the plant in most countries, it is performed on small scales or by illegal drug cultivators. In the previous part we already discussed the fact that cannabinoid production is mostly genetically determined. This knowledge could thus be used to increase the production of certain compound and decrease the other. Furthermore the total THC yield is dependent on the amount of accessible precursors and the level acceptable for the plant.
Within the *C. sativa* strain the total content of cannabinoids varies. In THCA dominant plants variations have also been noted. Some have low detectable amount of CBDA whereas others have none. Furthermore, some plants have shown to contain detectable amount of CBGA while others had none. It is still a question to what extend the THCA production can be increase in the plant by breeding programs and genetic modifications. From the genetic point of view it should be noted that the yield of THC is not only depending on the B_t allele, but

also depends on the amount of biomass, the density of trichomes and the production of precursors indicating a complex spectrum of different possibilities for professional plant breeders. The yield of THCA in THCA dominant plants can be increased by environmental influences. In cultivations of the 'drug'-type, mostly done by illegal cultivators, the male plant is excluded from the field. The background is that this will induce more biomass of the female plant because it can not be inseminated. But, the exclusion of male plants will not give a more constant increase of THC yield.

Genetic modification seems to be an option for increased yield since the THCA production is mainly dependent on genetic factors (see 3.1). Here we could think of increasing glandular trichoma densities, increase of precursor production, increase of enzyme activity and knocking out enzymes that use the general precursor of THCA (CBGA). Applying some of these techniques have already shown an increase in the amount of secondary metabolites in microbial organisms.

As stated above breeding programs could increase the total yield of THCA. Within *C. sativa* there are many phenotypes as discussed. While one has the ability to grow over a few meters the other stays small. Furthermore, variations in glandular trichoma densities have also been observed in THCA content and ratio's. By combining the phenotypes of various plants with each other, a plant could be grown that is large in growth, high in glandular trichoma density or high in THCA content. Through breeding techniques Meijer et. al. [56] already created a high CBGA producing plant and in the drug culture the same has been reached for THCA [61]. In the latter, preparations were found containing more than 20% of THC, while in literature and exported cannabis the normal values lays at 6-10%.

3.4.3 Cannabis standardisation

Just like all herbal medicinal preparations, *C. sativa* should be standardized, when extracts or whole plant material is used for medicinal purposes. Basic requirements are that all detectable constitutents should be known, but also a sustainable quality control system must be established to achieve the same quality over all batches. For industrial use of cannabis, standardization could also be necessary to equalize the quality of the product. However, it must be stated that cultivation for this purposes is mostly performed outdoors. Outdoor growth makes standardization of the product difficult due to

the environmental changes. For this reason the Dutch medicinal *C. sativa* is grown under strictly controllable conditions and thus indoor by the company Bedrocan b.v. At this company clones are used for breeding to maintain high standards for quantity and quality. After a strictly selective breeding procedure a plant line is established fulfilling all criteria as berb for medicinal use.

3.5 Alternative production systems for cannabinoids

It is clear that production of cannabinoids should be controllable to obtain a constant quality of certain cannabinoids. With the knowledge of the biosynthetic route towards cannabinoid production it is now possible to develop biological production systems as alternative to chemical synthesis. Major advantage of biological systems is not only of having the right natural product by structure, but also the only isomer in high yield. Here, three alternative production strategies are introduced. Although two of them are still hypothetical, it should be possible to be realize them in the near future.

3.5.1 Cell cultures

In literature several reports can be found on the growth of callus and cell suspension cultures [62-64]. Most of them document that no cannabinoids can be found within these cultures. Although one article by Heitrich and Binder [62] mentioned that variations in media can induce cannabinoid secretion, no second report could confirm these results. Callus and cell suspension are induced by standard techniques for plant cell manipulation. The induction of callus seems to vary per *C. sativa* variant [63]. To obtain cell suspensions from the callus, the same media is used as for callus growth with the exception of agar as solidifier. In literature, the cell suspensions made from C. sativa callus are mostly used for bioconversion studies. There is one report which described the use of cannabinoid precursors to determine if cannabinoid production can be induced by feeding with specific biosynthetic precursors [62]. When production of cannabinoids can be achieved in cell cultures from *C. sativa* material, it must still be considered that cannabinoids are toxic to the plant cell itself. Here theses compounds induce apoptotic response [23]. Thus, at high levels the cannabinoid content techniques has to be developed to extract them from growth media for continuous production.

3.5.2 Transgenic plants

Although the use of transgenic plants is not generally accepted for medicinal herbal preparations, transgenic plants could be used to express certain preferable traits. The THCA yield could be increased by manipulation of metabolic pathways or by making knock outs of biosynthetic genes. With the use of these techniques, the plant could be made resistant to certain parasites and diseases. General plant manipulating strategies can be used to obtain transgenic plants. There is no literature available for the production or use of transgenic *C. sativa* plants.
At the moment, strategies for the production of transgenic plants are already used in maize, tobacco, potato and rice plant. The main purpose is to increase their resistance toward diseases [65]. Some plants also get newly introduce products, such as vitamins [66]. Another purpose of transgenic plants is their use for production of vaccines; for instance hepatitis B vaccine in potato plants [67]. The examples shown here are a selection of many to show the possibilities transgenic plants uses.

3.5.3 Hetrologeous expression of cannabinoid biosynthetic genes.

Until now there are only two reports on heterologous expression of *C. sativa* origin genes into host organisms. In these reports the yeast *Pichia pastoris*, hairy root cultures of tobacco, BY-2 tobacco cell cultures and insect cells were used to produce the THCA synthase enzyme [40, 44]. In literature, the use of heterologous expression of plant metabolic enzymes have been shown to be useful in the production of several compounds [27]. The same strategy is probably useful for the production of cannabinoids. The production of cannabinoids will probably ask for specific cultivation parameters because some of its constituents may be toxic to the host in certain concentrations. One method could be a constant refreshment of the growth medium. Up today, no publications discuss the efforts of using the hetrologeous production of cannabinoid. This strategy could be, however, of high interests for Pharmaceutical companies, when some cannabinoids may be approved for medicinal use.

4 Chemical synthesis

4.1 Synthesis routes for Δ9-THC

After identification of Δ9-THC as the major active compound in *Cannabis* and its structural elucidation by Mechoulam and Gaoni in 1964 [68] a lot of work was invested in chemical synthesis of this substance. Analogous to the biosynthesis of cannabinoids the central step in most of Δ9-THC–syntheses routes is the reaction of a terpene with a resorcin derivate (e. g. olivetol). Many different compounds were employed as terpenoid compounds, for example citral [69], verbenol [70] or chrysanthenol [71]. The employment of optical pure precursors is inevitable to get the desired (-)-trans-Δ9-THC.

A general problem during the syntheses of Δ9-THC is the formation of the thermodynamically more stable Δ8-THC which one reduces the yield of Δ9-THC. It is formed from Δ9-THC by isomerisation under acidic conditions. While the usage of strong acids such as *p*-TSA or TFA leads to Δ8-THC mainly the yield of Δ9-THC can be increased by employment of weak acids, e. g. oxalic acid [72].

Recently mostly employed method for the production of Δ9-THC in industrial scale is the condensation of (+)-*p*-mentha-2,8-dien-1-ol (Fig. 5.1) with olivetol (Fig.5.2) in the presence of boron trifluoride etherate, $BF_3 \cdot OC(C_2H_5)_2$ with CBD as a key intermediate. This one-step synthesis of Δ9-THC is also used for the production of synthetic dronabinol which is used in the medicinal application named Marinol®. The mechanism of this synthesis is particular described by Razdan et. al. [73] and is shown in Fig. 5 containing the most important side products. There are two possibilities for the condensation of the active terpenoid moiety (Fig. 5.3) with activated olivetol (Fig. 5.4). The fusion of these compounds leads to two intermediates, normal CBD (Fig. 5.5) which has the same structure like natural CBD and ”abnormal” CBD (Fig.5.6) with transposed positions of the pentyl side chain and a hydroxy group. Fortunately is the latter compound less stable than the normal CBD and decompensate more easily. The normal CBD undergoes directly a further cyclisation to Δ9-THC (Fig. 5.7). If the double bond in the terpenoid ring is used for the cyclisation a isomeric compound named iso-tetrahydrocannabinol (iso-THC, Fig. 5.8) will be formed. The reaction has to be stopped here otherwise the stable isomer Δ8-THC (Fig. 5.9) is arising by decreasing the yield of Δ9-THC. Purification of the reaction mixture is implemented as a liquid chromatographic process using a silica-based stationary phase and a weak polar eluent

(e. g. heptane with 2% *tert*-butyl methyl ether). Further cleaning up is possible with vacuum destillation procedures.

Figure 5

4.2 Synthesis of Δ9-Tetrahydrocannabinol from natural Cannabidiol (semi-synthetic Δ9-THC)

As discussed the cultivation of *C. sativa* with high content of Δ9-THC (drug type) is not allowed in many countries. Out of this reason there is no opportunity to harvest a high amount the medicinal important substance Δ9-THC directly from plant material. In the synthesis route for semi-synthetic Δ9-THC natural CBD from fiber hemp plants is employed. It can be extracted with non polar solvents such as petroleum ether and purified by recrystallisation in n-pentane. This procedure avoids the formation of "abnormal" CBD and gives the opportunity to produce Δ9-THC from fiber hemp. Semi-synthetic Δ9-THC is distinguishably from the synthetic compound because it contains beside to the major product also small amounts of Δ9-THC-C3 and Δ9-THC-C4 which are not available in the synthetic product.

4.2.1 Derivates of Δ9-THC

Mostly relevant for the affinity for Δ9-THC and analogos to CB-receptors are the phenolic hydroxyl group at C-1, the kind of substitution at C-9 and the properties of the side chain at C-3. Relating to the structure-activity-relationships (SAR) between cannabinoids and the CB-receptors a lot of different modified structures of this substance group were developed and tested. The most important variations include variations of the side chain at the olivetolic moiety of the molecules and different substitutions at position C-11 respectively at C-9. One of the most popular analogous compounds of Δ9-THC is HU-210 or (-)-*trans*-11-OH-Δ8-THC-DMH, a cannabinoid with a 1',1,-dimethylheptyl side chain (Fig. 8.1). It was constructed in consideration of SAR and has a potency which is about 100 times high than this of Δ9-THC itself while its enantiomer HU-211 (Dexanabinol, Fig. 8.2) does not show this property [10]. In the synthesis of HU-210 5-(1,1-dimethylheptyl)-resorcin is merged with modified [1S,5R]-myrtenol [74].

Nabilone (Fig. 8.3) is a 11-nor-9-ketohexahydrocannabinoid with a 1',1'-dimethylheptyl side chain. It is a synthetic analogous compound of THC and is distributed as Cesamet® . Usage of diethyl-a-acetoglutarate as "terpenoid" module in the synthesis of Δ9-THC gives Nabilone as an intermediate [75]. In spite of the fact that this synthesis was developed for the forming of Δ9-THC it also could be used for the synthesis of Nabilone. A newer synthesis route is described by Archer et. al. [76]. The pentyl side chain homologous compound of Nabilone 11-nor-9-ketohexahydrocannabinol is a useful precursor in the chemical synthesis of the major metabolites of Δ9-THC, e. g. 11-nor-9-carboxy-Δ9-THC (THC-COOH) [77].

Direct oxidation of Δ9-THC at position C-11 involves mainly an isomerisation to Δ8-THC another opportunity in the synthesis of Δ9-THC-metabolites is the pretreatment of terpenoid synthons by introduction of protective groups, e. g. 1,3-dithiane (Fig. 6.1) followed by the condensation with olivetol (Fig. 6.2) [78]. The formed product is a protected derivate of Δ9-THC (Fig. 6.3) which will be modified furthermore. Protection of the phenolic group by esterification, for example is necessary before the removing of the 1,3-dithiane masking group with mercury oxide. The corresponding aldehyde (Fig. 6.4) can be further oxidized. The deprotection of the phenolic group by alkalic hydrolysis gives the 11-nor-9-carboxy-Δ9-THC (THC-COOH, Fig. 6.5). In case of using reductive conditions ($NaBH_4$ or $LiAlH_4$) the corresponding alcohol is formed from the aldehyde. This leads to 11-OH-THC (Fig. 6.6) which is the first major metabolite from Δ9-THC formed in humans [79].

Figure 6
Figure 7

In case of the employment of [^{2}H]-labeled precursors the resulting compounds can be used as internal standards for analysis especially by utilisation of mass spectrometric methods. Appropriate deuterated standards are shown in Fig. 7. The introduction of deuterium into the Δ9-THC precursors can be done with Grignard reagents such as C[$^2H_3$]MgI or reducing substances such as LiAl[$^2H_4$]. The general procedures for the synthesis with these [^{2}H]-labeled precursors are the same as described above for the unlabeled compounds [78, 80].

While as the compounds described above contain fundamentally the cannabinoidic structure there are also compounds with radical changes in, but still showing high affinity to CB-receptors. Exchange of oxygen with nitrogen in the pyran ring leads to a phenanthridine structure which can be found in levonantradol (Fig. 8.4). A compound with totally loss of the heterocyclic ring is CP-55,940 (Fig. 8.5). It can be comprehended as a disubstituted cyclohexanole and was synthesized by Pfizer in 1974. This compound was never marketed because of its high psychoactivity, but it is often used for CB-receptor binding studies [81]. Another group of multi-core chemical compounds based on the indol structure as a central module in these molecules shows also affinity to CB-receptors. The prototype of this class of aminoalkylindole cannabinoids is the substance named WIN 55,212-2 (Fig. 8.6), which is quite similar to pravadoline, an anti-inflammatory drug [82].

Figure 8

5 Analytics

5.1 Detection of cannabinoids in plant material

The chemical composition of *C.s sativa* is very complex and about 500 compounds in this plant are known. A complete list can be found in [83] with some additional supplementations [2, 84]. The complex mixture of about 120 mono- and sesquiterpenes is responsible for the characteristic smell of *C. sativa*. One of these terpenoic compounds, carophyllene oxide, is used as leading substance for hashish detection dogs to find *C. sativa* material [85]. It is a widespread error that dogs which are addicted to drugs are employed for drug detection. Δ9-THC is an odourless substance and can not be sniffed by dogs.

The aim of the analysis of cannabinoids in plants is to discriminate between the phenotypes (drug type / fiber type). Quantification of cannabinoids in plant material is needed if it will be used in medicinal applications, e. g. in *C. sativa* extracts. The ratio between Δ9-THC and CBN can be used for the determination of the age of stored marijuana samples [86].

5.1.1 Analytical methods for detection of Δ9-THC and other cannabinoids in plants

A lot of methods for determination of cannabinoids in plant material were developed. Commonly HPLC or GC is used, often in combination with mass spectrometry. Molecular techniques are also available to detect these compounds and will be discussed in this review.

Sample preparation

Usually the first step is an extraction of the desired compounds from plant material. This extraction can be done by different solvents, e. g. methanol [87], n-hexane [88], petroleum ether or solvent mixtures, e. g. methanol / chloroform [89]. The use of a second liquid-liquid-extraction (LLE) with 0.1M NaOH after extraction with a non-polar solvent like n-hexane makes a separate analysis of acidic cannabinoids possible which can be found as their salts in the water-phase [88]. These methods are useful for analysis of plant compartments like flowers or leaves, whereas for seeds a solid phase extraction (SPE) is preferred because of their very low content of cannabinoids [90]. The extracts are commonly used directly for analysis. In case of the analysis of acidic cannabinoids as like as they normally appear in plant material using GC-based methods a previous derivatisation of the analytes is commonly necessary.

Gas chromatographic methods (GC)

GC is commonly used for the analysis of cannabinoids, mostly in combination with mass spectrometry (GC-MS). Despite the fact that a lot of different cannabinoids are known almost all of them can be separated by using silica-fused non-polar columns. It is not possible to use GC-based methods for profiling of *C. sativa* samples. The high temperatures which are used in GC cause the decarboxylation of acidic cannabinoids. If an acidic cannabinoid such as THCA should be detected together with its neutral form such as Δ9-THC a derivatisation is required. This procedure increases the stability of the compounds whereas their volutility is maintained. The most often used reagents for derivatisation of cannabinoids in herbal samples are compounds which introduce trimethylsilyl groups (TMS) into the

analytes, for example *N,O-bis*(trimethylsilyl)trifuoroacetamide (BSTFA), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) or *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA). Furthermore mixtures of these compounds with catalysts, e. g. trimethylchlorosilane (TMCS) are used for a quantitative derivatisation [91]. Whileas the employment of established detectors such as flame ionisation detector (FID) or electron capture detector (ECD) can only give information about the quantity of a compound, the usage of mass spectrometry (MS) provides in addition information about the structures of detected compounds because of their characteristic fragmentation. For the quantification of cannabinoids often tri- or six fold and even tenfold deuterated compounds such as shown in Fig. 7.1 , 7.2 and 7.3 are used as internal standards. The fragmentation of cannabinoids in mass spectrometry is extensively explained by Harvey [92] and the interested reader can find there more informations about this topic. A table of about fifty cannabinoids cotaining free, derivated and deuterated compounds with their typical mass fragmentations is published by Raharjo and Verpoorte [91].

Liquid chromatographic methods (HPLC)

In comparison to GC an advantage in using HPLC is that there is no decomposition of the acidic forms of cannabinoids. Commonly reversed-phased (RP) materials are used as stationary phase. Mostly the octadecyl-type (C18) is employed. Furthermore the employment of a guard cartridge containing the same material as used as stationary phase is recommend normally. Typical mobile phases are mixtures of methanol and water or acetonitrile and water, acidified with phosphoric acid or formic acid. While for the separation of the main cannabinoids (Δ9-THC, CBD and CBN) an isocratic method is sufficient the separation of all cannabinoids makes a gradient elution necessary [89]. The use of a photodiode array detector (PDA) is recommend for identification of herbal cannabinoids because of their characteristic UV-spectra. If a PDA is used for the detection of cannabinoids Δ8-THC can be employed as an internal standard [93]. According to the law of Lambert-Beer a quantification of cannabinoids based on the strength of the absorption signal is possible. An excellent summary of the most important cannabinoids with their UV-spectra and other specific analytical data can be found in [94]. As like as described in the paragraph about GC-based methods the employment of mass spectrometry gives the opportunity to identify the structures combined with a better limit of detection (LOD) whereas the usage of an UV-detector lacks this sensitivity. Another

possibility structural identification gives the coupling of HPLC with NMR. The interpretation of [^{1}H]-signals which are specific for different substances also can be used for quantification [95].

Immunological based techniques

The Enzyme Linked Immuno Sorbent Assay (ELISA) technique is often used in laboratories for detection of proteins, but it is also possible to detect small organic molecules by this technique. This assay is based on antibodies which bind with high affinity to a certain molecular structures. Testing of cannabinoids by antibodies is already investigated since the seventies. The first detections were performed with radio labeled antibodies made by injection of conjugates from THC, its hemisuccinate and bovine serum albumin [96]. It was found that the antibody was able to detect cannabinoids and its metabolites from urine and plasma collected from rabbits administered with intravenous cannabinoids. In 1990, Elshoy et al. proved their antibodies to be specific to cannabinoids and related metabolites [97]. Furthermore, they tested against human cannabinoid metabolites excreted via urine and showed that the antibody against plant cannabinoids were also highly selective and did not bind to any of the non-cannabinoids phenolics. In the early days these studies were all performed with polyclonal antibodies, later monoclonal antibodies were tested and documented the same results [98, 99]. These antibodies may also be used for research. For instance, labeled antibodies have been used against the THC structures to determine that THC structures accumulate in the glandular trichoma. Moreover, with this technique it was possible to detect the specific place of accumulation within the trichoma [21]. This indicates that detection by antibodies has an added value to other detection methods, like HPLC and GC. It is thus possible to use these tests either with enzyme, fluorescent or radioactive labels to detect cannabinoids an its metabolites.

Molecular markers and PCR

These detection mechanisms are not able to detect the small organic structure of the cannabinoids. But these techniques are designed to make a selection between plant material on a genetic basis. For instance, by the use of only three polynucleotides (primers) and by the use of PCR discrimination of the major chemotypes as discussed above (see 3.1.2) was possible. Within the groups selected PCR

allowed 100% identification of the chemotypes without any cross reactivity [49]. Further more by simple PCR technique (2 primers used) a separation could be made between ‘drug type’ and ‘fiber type’ plants [46]. However, it must be stated that a very small number of plants was used and already polymorphism on the THCA synthase gene was found. The PCR technique can not be used to detect cannabinoids itself, but maybe it will be of value in plant breeding and cultivation. Furthermore it may find its place in the detection of illegal *C. sativa* (‘drug type’) within a population of legal (‘fiber type’) cultivated plants. However, for this technique, it would be convenient to have the genome of the *C. sativa* plant sequences. This would bring specific information of the differences between male and female plants and could make the design of markers for specific traits more easier.

5.2 Detection of Δ9-THC and its human metabolites in forensic samples

Δ9-THC and its main metabolites are detected and quantified in forensic samples. Determination of these compounds in human beings is needed to make decision on abuse of Δ9-THC containing drugs by individuals. A careful interpretation of the results is very important to avoid fallacies with regard to the behaviour of individuals. The *Cannabis* influence factor (CIF), for example, is an useful tool to distinguish between acute and chronic intake of Δ9-THC [100].

5.2.1 Metabolism of Δ9-THC by humane Cytochrome P450 enzymes

As other xenobiotics also cannabinoids undergo an extensive metabolism in the human body to increase their hydrophilic properties for a facilitated elimination. The metabolism of Δ9-THC has been investigated very well. More than 100 metabolites of Δ9-THC are known [101] and a good overview of the most important human metabolites is given in [102]. Metabolism takes place mainly in hepatic microsomes, but also in intestines, brain, heart, lung and nearly all tissues of the body. Main metabolites of Δ9-THC are mono-, di- and trihydroxylated compounds, which become carboxylated and glucuronidated furthermore. The metabolism pathway of Δ9-THC and its most important metabolites are shown in Fig. 9. Mostly responsible for metabolisation of Δ9-THC on primary pathway in humans is the

Cytochrome P450 isoenzyme CYP 2C9 [103]. Hydroxylation of Δ9-THC (Fig. 9.1) at C-11 leads to 11-hydroxy-Δ9-THC (11-OH-THC, Fig. 9.2) which undergoes further oxidation to 11-nor-9-carboxy-Δ9-THC (THC-COOH, Fig. 9.3). 11-OH-THC shows similar psychotropic properties to Δ9-THC whereas THC-COOH is a non psychotropic compound [104]. CYP 3A4 is the second major Cytochrome P450 isoenzyme which is involved in metabolism of Δ9-THC –mainly with the hydroxylation at C-8 to 8-OH-Δ9-THC, (Fig. 9.4) [103]. The epoxidation of Δ9-THC at C-9 and C-10 is also described, furthermore the oxidation of the alkyl side chain and a following cleavage [10]. Monohydroxylated Δ9-THC can be hydroxylated again which leads to 8,11-dihydroxy-Δ9-THC, (Fig. 9.5), for example. Metabolites which are formed by CYP 3A4 represent a minority in comparison with those of the CYP 2C9. The glucuronide of THC-COOH, (Fig. 9.6) which is formed on secondary pathway is a human metabolite of Δ9-THC.

Figure 9

5.2.2 Analytical methods for detection of Δ9-THC and it metabolites

As described for the analysis of the plant for the analysis of body fluids GC, HPLC, and immunoassays are commonly used. Although the general procedures are quite similar to those used in the analysis of *C. sativa* (see 5.2.1) some differences must be pointed out in the following part.

Sample preparation

The typical procedure for analysis of cannabinoids from plasma, urine or oral fluids includes preliminary steps such as a SPE for enhancement of the analytes and for minimizing interfering effects of the matrices. Because the metabolites in humans are often conjugated an anterior hydrolysis of these conjugates either with chemicals like sodium hydroxide or with enzymes [105] is recommend.
Pretreatments of hair samples also include an extraction, usually with an alkalic sodium hydroxide solution, followed by cleaning up with

LLE with *n*-hexane / ethyl acetate. Instead of LLE the employment of SPE is also possible. Furthermore the solid phase microextraction (SPME) in combination with head-space analysis is usable [106-108]. In case of using hair samples possible external contamination (e. g. by passive smoking of *Cannabis*) has to be considered as false positive result. False positive results can be avoided by washing of the hair samples previous extraction [109]. Storage of collected samples is another important fact which can cause false results in their content of Δ9-THC and metabolites [110-112].

Gas chromatographic methods (GC)

The preferred detection method for cannabinoids in forensic samples is GC-MS with or without preceding derivatisation. As described before in the analysis of plant materials the employment of silica-fused columns is recommend in the analysis of human body fluids. While in analysis of *Cannabis* TMS-reagents are mostly employed for derivatisation, in case of human body material as sample material fluoric compounds such as pentafluoropropionic anhydride (PFPA) or 2,2,3,3,3-pentafluoro-1-propanol (PFPOH) as derivatisation reagents are used [91]. Halogenation of the analytes in these ways give allow the use of an electron capture detector (ECD) to find desired compounds. In comparison with other detectors such as flame ionisation detector (FID) the detection sensivity of cannabinoids can be increased by using an ECD. This is important because the amount of these compounds is very low in human forensic samples. However, as mentioned above these detectors are commonly not used in routine analyses of forensic samples. Among PFPA and PFPOH also acylation reagents such as trifluoroacetic anhydride (TFAA) and *N*-methyl-*bis*(trifluoroacetamide) (MBTFA) are used for analysis of cannabinoids in human materials [113-116]. Trideuterated THC-COOH (Fig. 7.4) is the most common used internal standard for the analysis of metabolites with GC-MS. Baptista et. al. [105] have shown that the limit of quantification (LOQ) for the important metabolite THC-COOH is much more better in case of using the negative chemical ionisation (NCI) instead of electron ionisation (EI).

Liquid chromatographic methods (HPLC)

Whilst, for the analysis of plant material for cannabinoids both methods – GC and HPLC – are commonly used, in the analytical procedures prevails the employment of GC-based methods by far for

human forensic samples. Nonetheless the usage of HPLC becomes more and more of interest in this field especially in combination with MS [117-122]. Beside the usage of deuterated samples as internal standards Fisher et. al. [123] describe furthermore the use of a dibrominated THC-COOH (see Fig. 7.5). The usage of Thermospray-MS and electrochemical detection provide good performance and can replace the still used conventional UV detector. Another advantage in the employment of HPLC in contrast to GC could be the integration of SPE cartridges which are needed for sample preparation into the HPLC-system.

Immunoassays

Most of the tests which were developed for detection of cannabinoids in plants have shown that antibodies are specific for the cannabinoid structure. Because of this specifity these tests can be extensively applied for the detection of cannabinoids and metabolites in human body fluids such as plasma, urine and oral fluids. A lot of different kits based on these methods were developed and they are commercial available, for example Oratect®, Branan or Uplink®, OraSure. We must consider, however, that none of the humans have the same metabolite profile in their blood and that cross reactivity may always occur [124, 125]. Nevertheless, these tests offer a simple way of excluding most of the suspicious samples, but the results have to be still confirmed with a second method such as GC-MS [126, 127].

6 Medicinal use of *Cannabis* and cannabinoids

6.1 Historical aspects

Human use of *C. sativa* goes back over 10,000 years and the medicinal use can be definitely found in ancient Chinese writings from 1000 BC [128].Modern medicinal use was mainly introduced by William B. O'Shaugnessy who was one of the first physicians who systematically explored its therapeutic potential [129]. Studying the literature of the 19^{th} century it is impressive how efficiently most indications, which are now under intensive research, where already depicted by observation and simple trial and error.

6.2 Modern use

6.2.1 Natural cannabinoids

A serious problem in the early western medicinal use of *C. sativa*, mainly as a tincture, was its highly variable activity and inconsistent results. Medicinal preparations have to handle several particuliarities due to the structure of the active ingredients of *C. sativa*.

The identity of the main active constituent of *C. sativa*, Δ9-Tetrahydrocannabinol (INN Dronabinol) remained unknown until 1964 [130], standardized *C. sativa* preparation were not available.
Cannabinoids are highly lipophilic compounds making bioavailability very much depending on the formulation and the mode of administration.
The cannabinoids occurrence in the plant is predominantly in form of the carboxylic acids, which are pharmacologically totally different and rather unstable, decarboxylating over the time to their active neutral form.
The carboxylic acids, although not active at the CB receptor, nevertheless add to the overall effect, as they possess antibiotic and anti-inflammatory effects.
The plant itself is found in several different chemotypes, which added to the unpredictable nature of early medicinal preparations.
Last but not least the identification of THC as the main active constituent of *C. sativa* was preceeded by an almost total ban of the plant as a narcotic drug, practically ending medicinal research.

So, the 20th century actually led to an almost total disappearance of *C. sativa* for medicinal purpose. The only source for THC, which became the focus of scientific research was the rather tedious extraction and purification from confiscated Hashish or Marihuana. In 1972 the first commercially viable total synthesis of Δ9-THC was established and it became the first cannabinoid available as a modern medicine in the form of soft gel capsules (the active ingredient now being called Dronabinol from Tetrahy**dro**cann**abinol**) under the trade name Marinol® for the prevention of nausea and vomiting during cancer chemotherapy.
Interestingly this indication resulted from the observation of Marihuana smoking patients rather than from pharmacological research.

In contrast to the *C. sativa* tincture Marinol® soft gel capsules possess clear advantages. Firstly, they contain a single component in an accurate dosage. Secondly, it uses Sesame Oil as the carrier, making resorption significantly more reliable and also stabilizing the rather sensitive THC molecule.
The indication "prevention of nausea and vomiting during cancer chemotherapy" came from experiences of Marihuana smoking patients, not from pharmacological research [131].
The second indication being licensed for THC several years later came from an observation, which is known for a long time for *C. sativa*, namely its appetite stimulating effects. This sometimes very impressive effect (popularly also known as "munchies") was regarded as a side effect, until it became apparent, that loss of appetite and weight (the "AIDS wasting syndrome") was one of the determining factors influencing mortality of HIV patients [132].
Pharmacological research and the unprescriptional use of *C. sativa* by patients gave way to new indications. Well established now is the efficacy for the following indications:

Nausea and vomiting [131]
Appetite stimulation [133, 134]
Spasticity [135, 136]
Tourette syndrome [137]
Neuropathic pain [138]
Multiple sclerosis [139]
Mood elevation
Glaucoma [140]
Pruritus
Asthma
Epilepsia
Migraine

After the discovery of specific endocannabinoid receptors the amount of scientific literature quickly rose and not only new potential indications were established, but also mechanisms for the already known effects could be established. Although the most prominent effect of *C. sativa* is clearly related to THC and its activity at the CB1 receptor most other natural Cannabinoids are not active there. Today two other natural cannabinoids CBD and THCV are in the focus of medicinal research.

CBD was first isolated from *C. sativa* in 1940 [141]. Unlike the resinous air sensitive THC CBD is a crystalline stable substance. It's plant precursor, the carboxylic acid CBDA can be isolated from fiber

hemp by extraction and shows potent antibiotic activity. Upon heating it decarboxylates to CBD.
CBD has no activity at the CB1 or CB2 receptor. It is well known that CBD influences the activity of THC if coadministered [142]. Another effect of CBD is the inhibition of cytochrome oxidase [143], which inversely to its antagonistic activity strongly potentiates THC effects above a certain treshhold. CBD is also active as a mild antipsychotic [144] and was proposed as a treatment for anxiety and panic attacks. The mechanism is not fully understood, but it might be caused by an interference with the endocannabinoid system. It is now also under research for the treatment of diabetes and obesity [145].

6.2.2 Synthetic Cannabinoids

Until today only a few synthetic cannabinoids made its way into clinical use.

Nabilone

In contrast to THC (an oxygen sensitive resin) Nabilone [Fig. 8.3] is a crystalline stable substance. It is about 5 to 10 times more potent than THC [146]. It was developed by Lilly and marketed as Cesamed® in several countries, mainly for the prevention of nausea and vomiting during chemotherapy. Recently it was approved in the USA for the treatment of neuropathic pain.

Levonantradol

Levonantradol (Fig. 8.4) was synthesized with the intention to introduce a basic amino function into the heterocycle in the hope to get water soluble salts. Although the solubility of the hydrochloride is not good it was possible to get stable aqueos micellar solutions with the aid of emulsifiers [147] and it made its way as an incectable into clinical trials but never was approved.

CP -55,940

CP-55,940 (Fig. 8.5) was developed on the search for novel analgesics [148]. Although it is more potent than morphine it was never approved. Nevertheless, in it’s tritium labeled form it became a very

important tool for research and helped in the first identification of the cannabinoid receptor.

WIN-55,212-2

On the search for new anti inflammatory drug structurally derived from indomethacine [149], Pravadoline showed psychotropic side effects in clinical trials. It became apparent that these effects are mediated through the cannabinoid receptor. Optimization of the structure finally led to WIN-55,212-2 (Fig. 8.6) which has a higher affinity to the CB1 receptor than THC [150], which became an important research tool. The side effects of substances possessing agonistic activity on the CB1 receptor (mainly psychotropic effects like from cannabis) limiting it's clinical use changed the focus of research to the development of compounds without this drawback.

Rimonabant

Rimonabant or SR-141716A (Fig. 8.7) is an antagonist at the CB1 receptor [151] and got approval for the treatment of obesity and as an aid in the cessation of cigarette smoking. It is now marketed in Europe under the tradename Acomplia®. Interestingly the naturally occurring THCV (the propyl homologue of THC) also acts as an antagonist on the CB1 receptor and might become a competitor for rimonabant.

PRS 211,096

PRS 211,096 (Fig. 8.8) is a CB2 selective agonist thus avoiding the psychotropic side effects related to CB1. It is currently in clinical trial for the treatment of multiple sclerosis.

HU-211

HU-210 is (Fig. 8.1) among the most potent cannabinoids known. Its enantiomer HU-211 (Fig. 8.2) does not bind to the cannabinoid receptor and lacks psychotropic side effects (as long as optical purity is guaranteed). In animal models it shows analgesic and antiemetic activity. It also shows neuroprotective effects after brain injury and was tested in humans as anti-trauma agent, where it did not meet the expectations in a clinical phase III trial.

Ajulemic acid

Ajulemic acid (CT3, Fig 8.9) is of the dimethylheptyl homologue of the main metabolite of Δ8-THC. It has no psychotropic activity, but has analgetic and anti inflammatory effects.

6.3 Drug Delivery

The classical way of application of Δ9-THC from *C. sativa* is smoking of dried *Cannabis* flowers or leaves by patients in traditional medicine. Smoking of *Cannabis* as an illegal drug is popular, but not only these drug users but also regular patients suffering from varies diseases as discussed above use this form of unprescribed self-medication.

Besides of the inhalative use the development of a drug formulation for Δ9-THC has to address also other bioavailability question. A major problem is the lipohilicity and poor solubility in water limiting the oral uptake when given orally. Out of this reason other parenteral routes of application are under investigation like pulmonal uptake by vaporization, sublingual or intranasal administration, and application by injection of Δ9-THC incorporated in liposomes.

Marinol® and Sativex® are given orally to the patient, but as indicated the poor solubility of Δ9-THC is responsible for its slow onset and release from drug carriers like soft gelatine capsules [152], for quite frequently a large variety in the bioavailability and a significant first pass effect can be observed in animal tests and patients. One solution to the solubility problem is the development of new Δ9-THC derivatives with improved solubility like dexanabinol, what is a hemisuccinate prodrug, but this strategies are mostly not desirable because of the high risk to go again through the cost and time consuming drug approval process to gain all toxicological and clinical data.

Main strategies to improve solubility in Pharmaceutical Technology is the reduction of particle size and increasing of particle surface according to the Kelvin equitation. Two strategies has been applied for Δ9-THC by production by solid dispersion technology and production of nanosuspensions. Van Drooge et al., 2004, created a

solid dispersion of inulin in which Δ9-THC was incorporated [153]. Applying freeze drying technique for evaporation of a mixture of water and tertiary butyl alcohol remaining, which act as dissolving medium for Δ9-THC and inulin, will form amorphous Δ9-THC in a fast dissolving solid inulin matrix. Main advantage of the technique is to protect Δ9-THC from degeneration and to optimize the dissolution rate from tablets [153]. A second and easy way to increase the solubility can be achieved by reduction of the particle size. In unpublished work by the authors group nanosuspensions of Δ9-THC has been achieved indicating a first significant improvement on this physical properties. Main drawbacks of the technique is the poor stability of the high energetic suspensions and the risk to form cluster and microparticles without sufficient stabilization of the nanosuspension. Perlin et al., 1985, applied Δ9-THC incorporated in gelatine capsules, and administered these orally to rhesus monkeys at a dose of 2.5-mg/kg doses and compared the plasma levels with parenteral intravenous and intramuscularly injections [154]. The authors conclude their study that intramuscularly injection is in favour because of bioavailability of 89 % ± 16% (i.m.) versus 26 % ± 14 % (p.o.). Interestingly Perlin et al., 1985, mentioned that rectal administration was not successful and no significant blood levels were detected [154]. More recently Munjal et al., 2006, developed a transmucosal system based on polyethylene oxid (PEO) polymers which are commonly used for the production of suppositories [152]. In this study the heat labile Δ9-THC hemisuccinate was used to produce suppositories varying in the PEO composition by the hot-melt technique (120 °C). Temperature led to a degradation between 13.5 % and 49.4 % depending on the composition, but incorporation of vitamine E succinate reduced processing degradation to 9.2 % and a shelf half life time of 8 month. No data have been published yet to characterize the bioavailability or pharmacological effect.

To achieve reliable elevated plasma levels and to overcome first pass effect alternative parenteral administration systems have been developed. The most obvious route is vaporization of the *Cannabis* plant material or the Δ9-THC directly. Hazekamp et al., 2005, conducted an intensive study using the Volcano device [155]. Main principle is evaporation of Δ9-THC from Cannabis plant material by a hot air flow. Evaporated compounds are collected in a detectable plastic balloon, which can be removed and fitted with a mouthpiece for inhalation. Main advantage of the Volcano vaporisator is that Δ9-THC is vaporized below the point of combustion avoiding the production of lung irritating toxins. Other advantages for the self medicating patient is the ease of self titration, fast drug release and

fast reaching therapeutic blood levels. In comparison to alternative smoking procedures including water pipe the Δ9-THC recovery was for the Volcano 54% and for the water pipe 39%, respectively.

Pulmonal application can be still unpleasant for non smokers why other administration routes like sublingual or intranasal uptake are also of interest. Valiveti et al., 2007, investigated nasal application for Δ9-THC and WIN55,121-2 mesylate in rates [156]. Last one is a synthetic cannabinoid with a short half life time and a high variable bioavailability. Both drugs were formulated in ethanol and propylene glycol and were successfully administered. In comparison with i.v. applied reference drugs a ten times higher nasal dose (10 mg/kg Δ9-THC) showed similar AUC values with a slightly increased half life time.

A second alternative is sublingual application as introduced by Mannila et al., 2005, based on cyclodextrinmatrices [157]. Cyclodextrins are a group of cyclic oligosaccharides, which have been shown to improve aqueous solubility, dissolution rate and bioavailability of various lipophilic drugs like testosterone or prostaglandin E to give two examples. Cyclodextrines have also been successfully studied in a few sublingual and buccal formulations, e.g. hydroxypropyl-β-cyclodextrin (HP-β-CD) led to the effective absorption of sublingual testosterone.
In this study complexation of Δ9-THC and cannabidiol, with randomly methylated β-cyclodextrin and hydroxypropyl-β-cyclodextrin (HP-β-CD), were studied by the phase-solubility method which were prepared by freeze drying. The aqueous solubility of CBD and THC increased as a function of CD concentration, and the dissolution increased for THC and CBD cyclodextrin complexes significantly in contrast to plain THC and CBD. These results demonstrate that cyclodextrines increased both the aqueous solubility and dissolution rate of these cannabinoids, making the development of novel sublingual formulation possible what has been shown by in vivo studies in New Zealand rabbits.

Table 1
Chemical and physical properties of *(-)-trans*-Δ9-THC [4-6]

Molecular weight	314.47
Molecular formula	$C_{21}H_{30}O_2$
Boiling point	200°C (at 0.02mm Hg)
Rotation of polarized light	$[a]^{20}_D$ = -150.5° (c = 0.53 in $CHCl_3$)
UV maxima	275nm and 282nm (in ethanol)
Mass fragments (m/z)[a]	314 (M+); 299; 271; 258; 243; 231
pK_a	10.6
Stability	not stable in acidid solution
	($t_{1/2}$ = 1h at pH 1.0 and 55°C)
Partition coefficient	12091
(octanol/water)[b]	highly insoluble in water (~ 2.8mg per L at
Solubility	23°C)
	highly soluble in methanol, ethanol

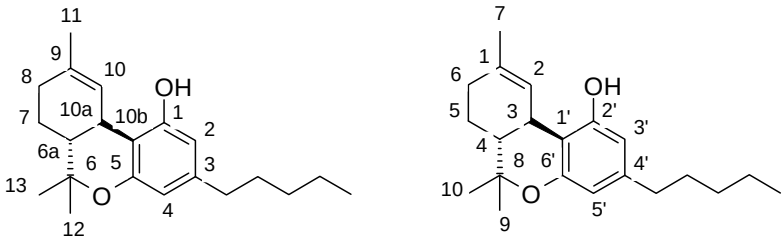
[a] These mass fragments were found by own measurement
[b] In the literature values between 6000 and 9 440 000 can be found [104]

Table 2
Enzyme properties from enzymes found in the cannabinoid biosynthesis

Enzyme	**pH optimal/pI**	**Reaction rate (in vitro) $k_{cat}=s^{-1}$**	**localization**	**Metal ion**	**Cofactors**	Mw (obtain from protein isolation, not heterologous expressed)	comments	substrates	refs
GOT	7/N.R	N.R	?	Mg^{2+}				Olivetolic acid with GPP or NPP	[34]
CBDA	5/6.1	0.19[a]	?**	None	None*	~75kDa		CBGA and CBNRA	[41]
THCA	7.1/6.4	0.2[a]-0.3[b]	storage cavity of glandular trichome	None	FAD*	~74kDa		CBGA and CBNRA	[40, 43]
CBCA	6.5/7.1	0.04[a]	?	none	None*	~71kDa	Probably homodimeric	CBGA and CBNRA	[42]

Table 2 continued

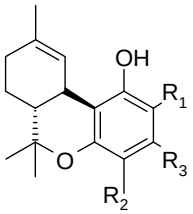
*= Activity test with crude extract did not show the need for cofactors, however from analysis performed on THCA synthase, it became clear hat FAD is co-valently bound to the enzyme. Furthermore the analysis of the enzymes THCA and CBDA showed the motif that in conserved for FAD binding.
**=CBDA synthase shown to carry a highly similar N-terminal signal sequence compared to THCA-synthase. It is thus suggested that this enzyme is localized at the same position as THCA synthase. Furthermore the precursor CBGA have been shown to be toxic for plant cells and is probably localized in the secretory cavity of the glandular trichome, thus it could be suggested that CBDA, THCA, CBCA are all localized in the storage cavity.
N.R=not reported.
[a]= determined by purified Cannabis extract
[b]= determined by recombinant proteins isolates



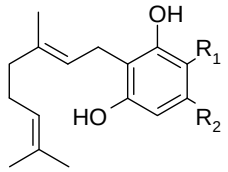
(1.1) Dibenzopyrane numbering system

(1.2) Terpene numbering system

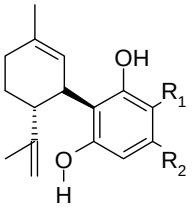
Fig. 1 Commonly used numbering systems for cannabinoids



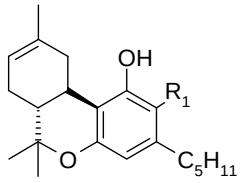
(2.1) R_1=H R_2=H R_3=C_5H_{11}
(2.2) R_1=COOH R_2=H R_3=C_5H_{11}
(2.3) R_1=H R_2=COOH R_3=C_5H_{11}
(2.4) R_1=H R_2=H R_3=C_3H_7
(2.5) R_1=H R_2=H R_3=C_4H_9



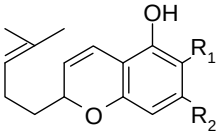
(2.6) R_1=H R_2=C_5H_{11}
(2.7) R_1=COOH R_2=C5H_{11}
(2.8) R_1=H R_2=C_3H_7



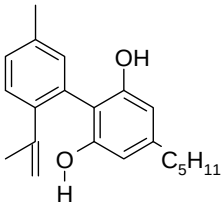
(2.9) R_1=H R_2=C_5H_{11}
(2.10) R_1=COOH R_2=C_5H_{11}
(2.11) R_1=H R_2=C_3H_7
(2.12) R_1=H R_2=C_4H_9



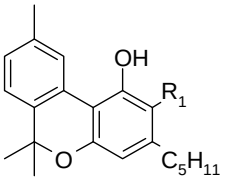
(2.13) R_1=COOH
(2.14) R_1=H



(2.15) R_1=H R_2=C_5H_{11}
(2.16) R_1=COOH R_2=C_5H_{11}
(2.17) R_1=H R_2=C_3H_7



(2.18)



(2.19)

Figure 2. Chemical structures of some natural cannabinoids

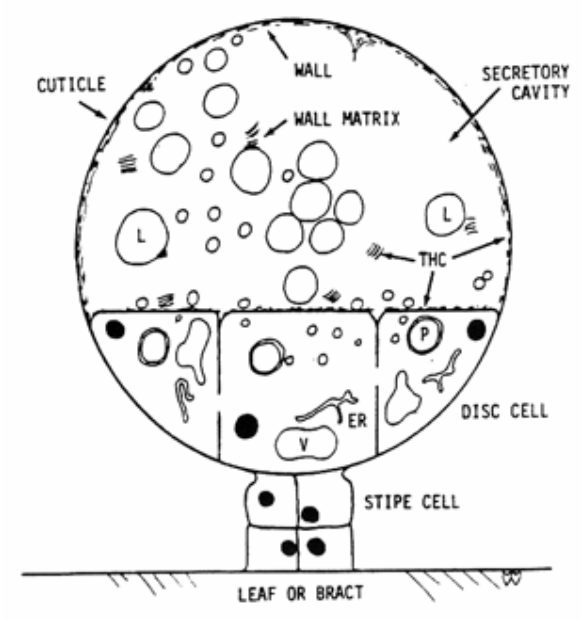


Fig. 3. A representation of mature secretory gland originated from C.sativa. within this picture the separate compartments of the glandular trichome are clearly shown. Moreover the place were THC accumulates are given. Nucleus = black; vacuole = V; vesicles = L; plastids = P; endoplasmic reticulum = ER. Picture obtained from: http://www.hempreport.com/issues/17/malbody17.html

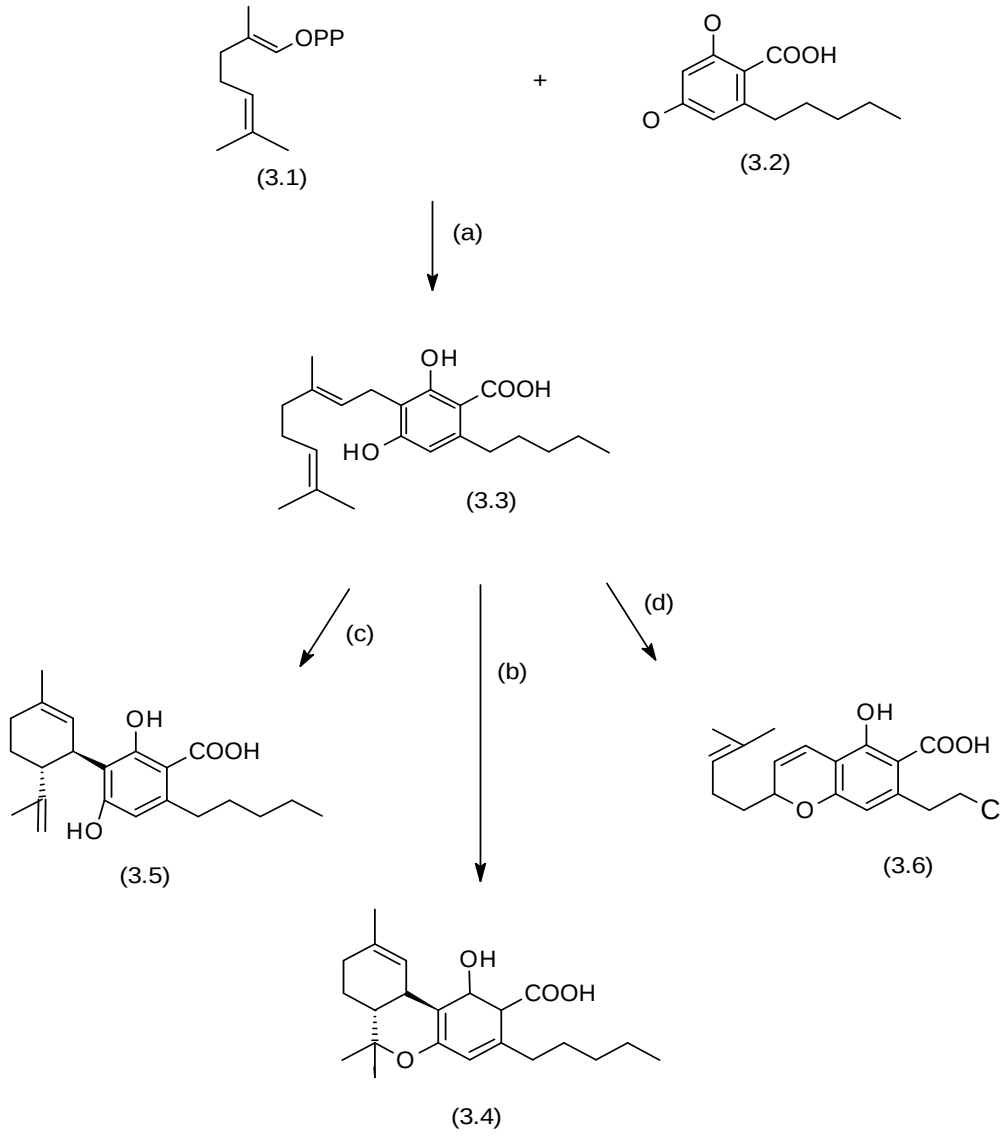


Fig. 4. Biosynthesis of THC and related cannabinoids. (a) GOT ; (b) THCs ; (c) CBDs ; (d) CBCs

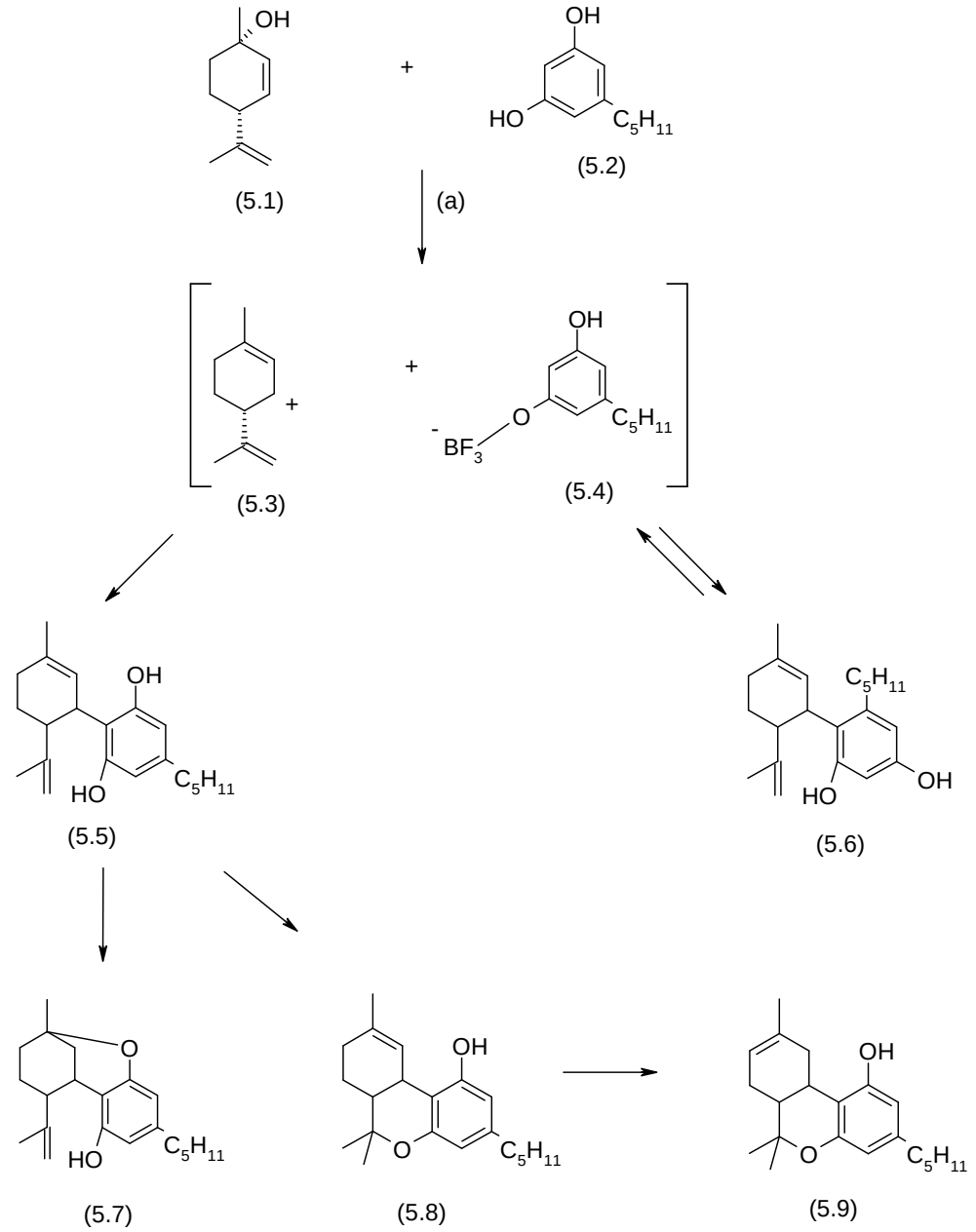


Fig. 5. Commonly used synthesis of D9-THC
(a) $BF_3{\cdot}O(C_2H_5)_2$/DCM/Mg_2SO_4

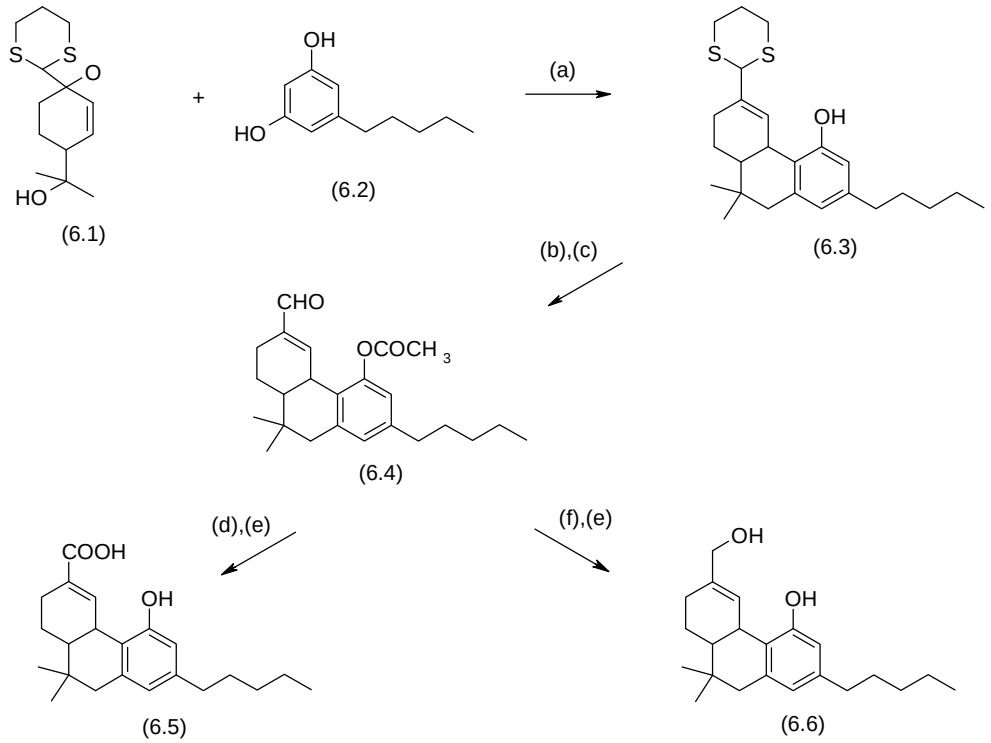
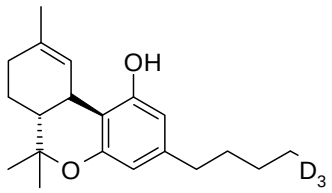
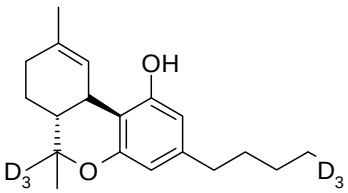


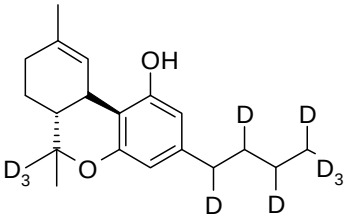
Fig. 6. Synthesis of main metabolites of D9-THC
(a) CH3SO3H ; (b) (C2H5O)2O/pyridine ; (c) HgO/BF3.O(C2H5)2 ;
(d) NaCN/CH3COOH/MnO2 ; (e) NaOH/THF ; (f) NaBH4/EtOH



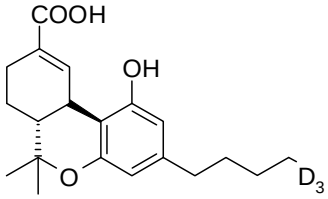
(7.1)



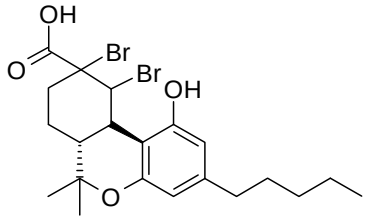
(7.2)



(7.3)



(7.4)



(7.5)

Fig. 7. Deuterated and brominated cannabinoids as internal analytical standards

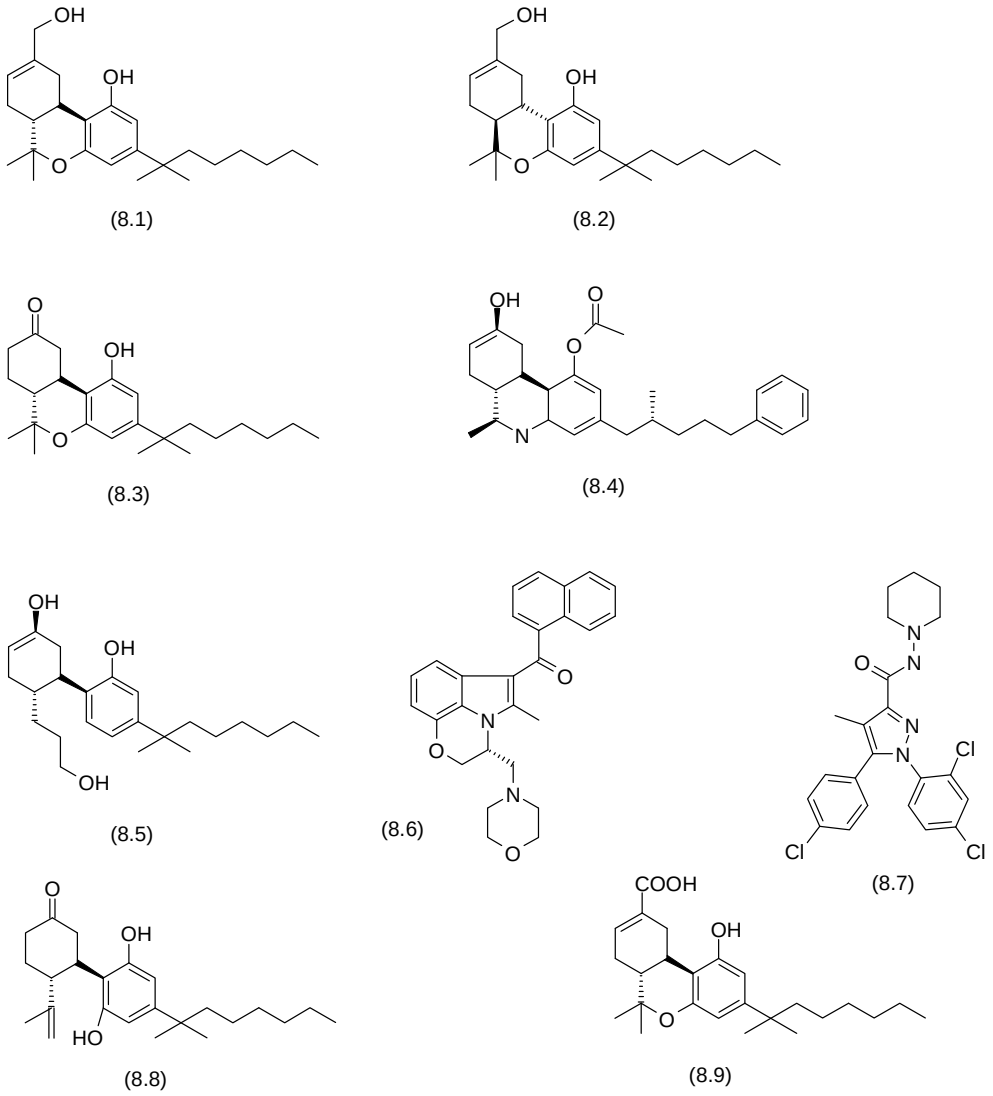


Fig. 8. Synthetic derivatives of Δ9-THC

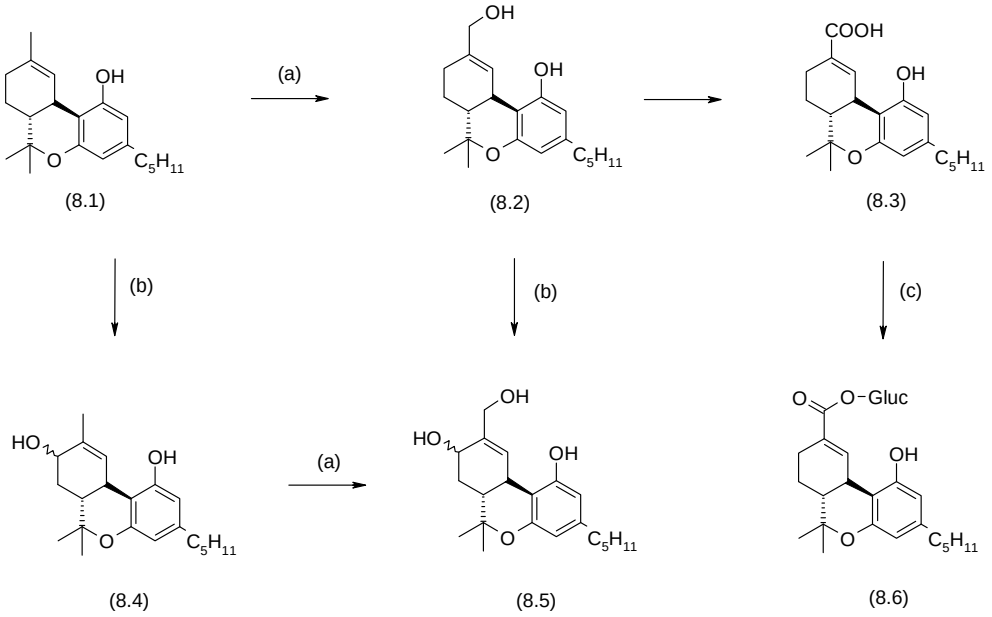


Fig. 9. Main metabolism pathway of D9-THC in humans
(a) CYP 2C9 ; (b) CYP 3A4 ; (c) UGT

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