

Chemistry and Biological Activity of Tetrahydrocannabinol and its Derivatives

T. Flemming^{1,2} · R. Muntendam² · C. Steup¹ · Oliver Kayser³ (✉)

¹THC-Pharm Ltd., Offenbacher Landstrasse 368A, 60599 Frankfurt, Germany

²Department of Pharmaceutical Biology, GUIDE, University of Groningen,
Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands

³Department of Pharmaceutical Biology,
Groningen Research Institute for Pharmacy (GRIP), University of Groningen,
Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands
o.kayser@rug.nl

T. Flemming and R. Muntendam both contributed equally

1	Chemistry	2
1.1	Nomenclature	2
1.2	Chemical and Physical Properties of Δ^9 -THC	3
1.3	Further Natural Cannabinoids	5
1.3.1	Cannabigerol (CBG)	5
1.3.2	Cannabidiol (CBD)	6
1.3.3	Δ^8 -Tetrahydrocannabinol (Δ^8 -THC)	6
1.3.4	Cannabichromene (CBC)	6
1.3.5	Cannabinodiol (CBND) and Cannabinol (CBN)	7
2	Biosynthesis of Cannabinoids	7
2.1	Biochemistry and Biosynthesis	8
2.2	Genetics of <i>Cannabis Sativa</i>	13
2.3	Environmental Factors	15
2.3.1	Dehydration	15
2.3.2	Nutrients in Soil	15
2.3.3	Light	15
2.4	Growing of <i>Cannabis Sativa</i> and Optimization of THC Yield	16
2.4.1	Cultivation of <i>Cannabis</i>	16
2.4.2	Optimization of THC Yield	16
2.4.3	<i>Cannabis</i> Standardization	17
2.5	Alternative Production Systems for Cannabinoids	17
2.5.1	Cell Cultures	18
2.5.2	Transgenic Plants	18
2.5.3	Heterologous Expression of Cannabinoid Biosynthetic Genes	19
3	Chemical Synthesis	19
3.1	Synthesis Routes for Δ^9 -THC	19
3.2	Synthesis of Δ^9 -Tetrahydrocannabinol from Natural Cannabidiol (Semisynthetic Δ^9 -THC)	21
3.2.1	Derivates of Δ^9 -THC	21

4	Analytics	25
4.1	Detection of Cannabinoids in Plant Material	25
4.1.1	Analytical Methods for Detection of Δ^9 -THC and Other Cannabinoids in Plants	25
4.2	Detection of Δ^9 -THC and its Human Metabolites in Forensic Samples	28
4.2.1	Metabolism of Δ^9 -THC by Humane Cytochrome P450 Enzymes	28
4.2.2	Analytical Methods for Detection of Δ^9 -THC and it Metabolites	29
5	Medicinal use of <i>Cannabis</i> and Cannabinoids	31
5.1	Historical Aspects	31
5.2	Modern Use	32
5.2.1	Natural Cannabinoids	32
5.2.2	Synthetic Cannabinoids	34
5.3	Drug Delivery	35
	References	38

Abstract Cannabinoids and in particular the main psychoactive Δ^9 -THC are promising substances for the development of new drugs and are of high importance in biomedicine and pharmacy. This review gives an overview of the chemical properties of Δ^9 -THC, its synthesis on industrial scale, and the synthesis of important metabolites. The biosynthesis of cannabinoids in *Cannabis sativa* is extensively described in addition to strategies for optimization of this plant for cannabinoid employment in medicine. The metabolism of Δ^9 -THC in humans is shown and, based on this, analytical procedures for cannabinoids and their metabolites in human forensic samples as well as in *C. sativa* will be discussed. Furthermore, some aspects of medicinal indications for Δ^9 -THC and its ways of administration are described. Finally, some synthetic cannabinoids and their importance in research and medicine are delineated.

Keywords Tetrahydrocannabinol · *Cannabis sativa* · Analytical methods · Medicinal applications

1

Chemistry

1.1

Nomenclature

Natural cannabinoids are terpenophenolic compounds that are only biosynthesized in *Cannabis sativa* L., Cannabaceae. For these compounds five different systems of nomenclature are available, well described by Shulgin [1] and by ElSohly [2]. Two of these systems are mainly employed for the description of tetrahydrocannabinol in publications – the dibenzopyrane numbering system (1.1 in Fig. 1) and the terpene numbering system (1.2), based on *p*-cymene. Because of historical and geographical reasons, the missing standardization is not uniform and is the main reason for ongoing confusion in the literature, leading to discussions regarding the numbering and

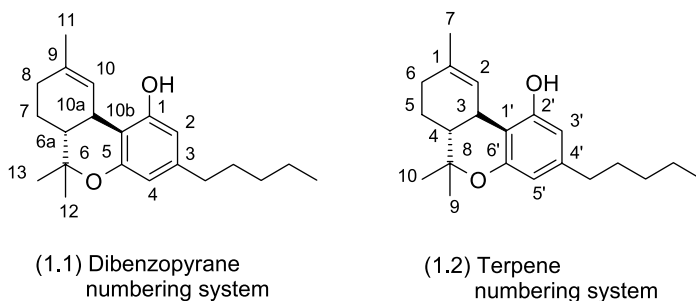


Fig. 1 Commonly used numbering systems for cannabinoids

its order. As an example, the use of the terpene numbering system gives the name Δ 1-tetrahydrocannabinol; in contrast, using the dibenzopyrane numbering system leads to the name Δ 9-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 this system has a general disadvantage because of a complete change in numbering after loss of the terpenoid ring, as found in many cannabinoids.

The chemical name of Δ 9-THC according to the dibenzopyrane numbering system is 3-pentyl-6,6,9-trimethyl-6*a*,7,8,10*a*-tetrahydro-6*H*-dibenzo-*[b, d]*pyran-1-ol as depicted in 1.1 (Fig. 1).

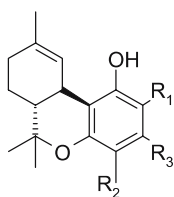
Alternatively, Δ 9-tetrahydrocannabinol or simply tetrahydrocannabinol is frequently used in the scientific community. When using the short name tetrahydrocannabinol or just THC it always implies the stereochemistry of the Δ 9-isomer.

On the market are two drugs under the trade names of Dronabinol, which is the generic name of *trans*- Δ 9-THC, and Marinol, which is a medicine containing synthetic dronabinol in sesame oil for oral intake, distributed by Unimed Pharmaceuticals.

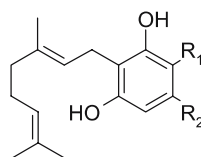
1.2

Chemical and Physical Properties of Δ 9-THC

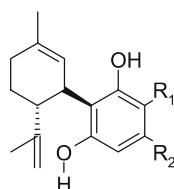
Δ 9-THC (2.1 in Fig. 2) is the only major psychoactive constituent of *C. sativa*. It is a pale yellow resinous oil and is sticky at room temperature. Δ 9-THC is lipophilic and poorly soluble in water ($3 \mu\text{g mL}^{-1}$), with a bitter taste but without smell. Furthermore it is sensitive to light and air [4]. Some more physical and chemical data on Δ 9-THC are listed in Table 1. Because of its two chiral centers at C-6*a* and C-10*a*, four stereoisomers are known, but only (-)-*trans*- Δ 9-THC is found in the *Cannabis* plant [5]. The absolute configuration of the



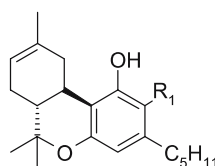
- (2.1) $R_1=H$ $R_2=H$ $R_3=C_5H_{11}$
 (2.2) $R_1=COOH$ $R_2=H$ $R_3=C_5H_{11}$
 (2.3) $R_1=H$ $R_2=COOH$ $R_3=C_5H_{11}$
 (2.4) $R_1=H$ $R_2=H$ $R_3=C_3H_7$
 (2.5) $R_1=H$ $R_2=H$ $R_3=C_4H_9$



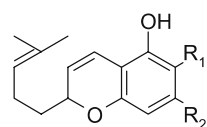
- (2.6) $R_1=H$ $R_2=C_5H_{11}$
 (2.7) $R_1=COOH$ $R_2=C_5H_{11}$
 (2.8) $R_1=H$ $R_2=C_3H_7$



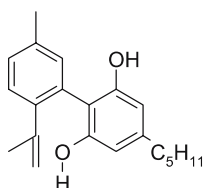
- (2.9) $R_1=H$ $R_2=C_5H_{11}$
 (2.10) $R_1=COOH$ $R_2=C_5H_{11}$
 (2.11) $R_1=H$ $R_2=C_3H_7$
 (2.12) $R_1=H$ $R_2=C_4H_9$



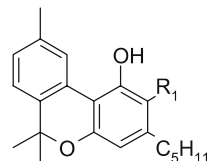
- (2.13) $R_1=COOH$
 (2.14) $R_1=H$



- (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)

Fig. 2 Chemical structures of some natural cannabinoids

natural product was determined as (*6aR,10aR*) [6]. Depending on the position of the double bond in the terpenoid ring six isomers are possible, whereof the $\Delta 9$ -isomer and the $\Delta 8$ -isomer are most important. Conformational studies of $\Delta 9$ -THC using NMR techniques were done by Kriwacki and Makryiannis [7]. The authors found that the arrangement of the terpenoid ring and pyrene ring of this compound is similar to the half-opened wings of a butterfly. An excellent

Table 1 Chemical and physical properties of (-)-*trans*- Δ 9-THC [4]

Molecular weight	314.47
Molecular formula	C ₂₁ H ₃₀ O ₂
Boiling point	200 °C (at 0.02 mm Hg)
Rotation of polarized light	[α] _D ²⁰ = - 150.5° (<i>c</i> = 0.53 in CHCl ₃)
UV maxima	275 nm and 282 nm (in ethanol)
Mass fragments (<i>m/z</i>) ^a	314 (M ⁺); 299; 271; 258; 243; 231
pK _a	10.6
Stability	Not stable in acidic solution (<i>t</i> _{1/2} = 1 h at pH 1.0 and 55 °C)
Partition coefficient (octanol/water) ^b	12 091
Solubility	Highly insoluble in water (~ 2.8 mg L ⁻¹ at 23 °C)

^a These mass fragments were found by our own measurements

^b In the literature, values between 6000 and 9 440 000 can be found [102]

review by Mechoulam et al. has been published providing more information on this topic and discussing extensively the stereochemistry of cannabinoids and Δ 9-THC, with special focus on the structure–activity relationship [8].

It must be noted that Δ 9-THC is not present in *C. sativa*, but that the tetrahydrocannabinolic acid (THCA) is almost exclusively found. Two kinds of THCA are known. The first has its carboxylic function at position C-2 and is named 2-carboxy- Δ 9-THC or THCA-A (**2.2**); the second has a carboxylic function at position C-4 and is named 4-carboxy- Δ 9-THC or THCA-B (**2.3**).

THCA shows no psychotropic effects, but heating (e.g., by smoking of *Cannabis*) leads to decarboxylation, which provides the active substance Δ 9-THC. Δ 9-THC is naturally accompanied by its homologous compounds containing a propyl side chain (e.g., tetrahydrocannabivarin, THCV, THC-C₃, **2.4**) or a butyl side chain (THC-C₄, **2.5**).

1.3

Further Natural Cannabinoids

Seventy cannabinoids from *C. sativa* have been described up to 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 Δ 9-THC and their use in medicinal applications.

1.3.1

Cannabigerol (CBG)

Cannabigerol (CBG, **2.6**) was historically the first identified cannabinoid [9]. It can be comprehended as a molecule of olivetol that is enhanced with

2,5-dimethylhepta-2,5-diene. In plants, its acidic form cannabigerolic acid (CBGA, 2.7) and also the acid forms of the other cannabinoids prevail. CBGA is the first cannabinoidic precursor in the biosynthesis of Δ^9 -THC, as discussed in Sect. 2. Although the *n*-pentyl side chain is predominant in natural cannabinoids, cannabigerols with propyl side chains (cannabigerovarin, CBGV, 2.8) are also present.

1.3.2

Cannabidiol (CBD)

The IUPAC name of cannabidiol is 2-[(1*S*, 6*R*)-3-methyl-6-prop-1-en-2-yl-1-cyclohex-2-enyl]-5-pentyl-benzene-1,3-diol. Cannabidiol (CBD, 2.9) in its acidic form cannabidiolic acid (CBDA, 2.10) is the second major cannabinoid in *C. sativa* besides Δ^9 -THC. As already mentioned for Δ^9 -THC, variations in the length of the side chain are also possible for CBD. Important in this context are the propyl side chain-substituted CBD, named cannabidivarin (CBDV, 2.11), and CBD-C₄ (2.12), the homologous compound with a butyl side chain. Related to the synthesis starting from CBD to Δ^9 -THC as described in Sect. 3.1, 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 that it is unlikely that the biosynthesis of THCA from CBDA takes place in *C. sativa*.

1.3.3

Δ^8 -Tetrahydrocannabinol (Δ^8 -THC)

This compound and its related acidic form, Δ^8 -tetrahydrocannabinolic acid (Δ^8 -THCA, 2.13) are structural isomers of Δ^9 -THC. Although it is the thermodynamically stable form of THC, Δ^8 -THC (2.14) contributes approximately only 1% to the total content of THC in *C. sativa*. In the synthetic production process, Δ^8 -THC is formed in significantly higher quantities than in plants.

1.3.4

Cannabichromene (CBC)

Among THCA and CBDA, cannabichromene (CBC, 2.15) and the acidic form cannabichromenic acid (CBCA, 2.16) are formed from their common precursor CBGA. Besides CBC, its homologous compound cannabiverol (CBCV, 2.17) with a propyl side chain is also present in plants.

1.3.5

Cannabinodiol (CBND) and Cannabinol (CBN)

Cannabinodiol (CBND, 2.18) and cannabinol (2.19) are oxidation products of CBD and Δ^9 -THC formed by aromatization of the terpenoid ring. For the dehydrogenation of THC a radical mechanism including polyhydroxylated intermediates is suggested [10, 11]. CBN is not the sole oxidation product of Δ^9 -THC. Our own studies at THC-Pharm on the stability of Δ^9 -THC have shown that only about 15% of lost Δ^9 -THC is recovered as CBN.

2

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 [12]. 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% (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 Sect. 2.2), in the 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 for its tempering effect on the symptoms of multiple sclerosis [13], but CBG and CBD may also have a role in medicine. Both CBD and CBG are related to analgesic and anti-inflammatory effects [14, 15].

In this section, the latest developments and recent publications on the biosynthesis of Δ^9 -THC and related cannabinoids as precursors are discussed. Special points of interests are the genetic aspects, enzyme regulation, and the environmental factors that have an influence on the cannabinoid content in the plant. Because of new and innovative developments in biotechnology we will give a short overview of new strategies for cannabinoid production in plant cell cultures and in heterologous organisms.

2.1 Biochemistry and Biosynthesis

The biosynthesis of major cannabinoids in *C. sativa* is located in the glandular trichoma, which are located on leaves and flowers. Three known resin-producing glandular trichoma are known, the bulbous glands, the capitate sessile, and the capitate stalked trichoma. It has been reported that the latter contain most cannabinoids [16]. The capitate stalked trichoma become abundant on the bracts when the plant ages and moves into the flowering period. The capitate sessile trichoma show highest densities during vegetative growth [17, 18].

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 [17–21]. Here, Δ^9 -THC was found to accumulate in the cell wall, the fibrillar matrix and the surface feature of vesicles in the secretory cavity, the subcuticular wall, and the cuticula of glandular trichomes [19].

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 ter-

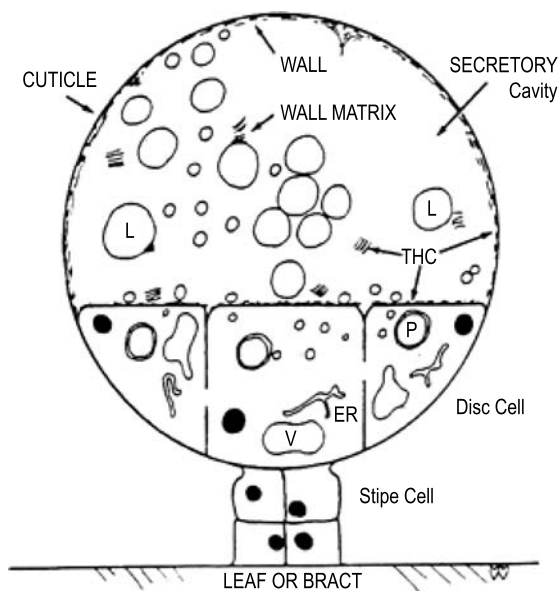


Fig. 3 Representation of mature secretory gland originated from *C. sativa*. The separate compartments of the glandular trichome are clearly shown, and the places where THC accumulates. *Black areas* nuclei, *V* vacuole, *L* vesicle, *P* plastid, *ER* endoplasmic reticulum. Picture obtained from: <http://www.hempreport.com/issues/17/malbody17.html>

penoid production is already reviewed exhaustively [22–25]. The phenolic unit of cannabinoids is thought to be produced via the polyketide pathway [26–28]. 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 discuss 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). For convenience the abbreviations of the acidic form will be used through this section because

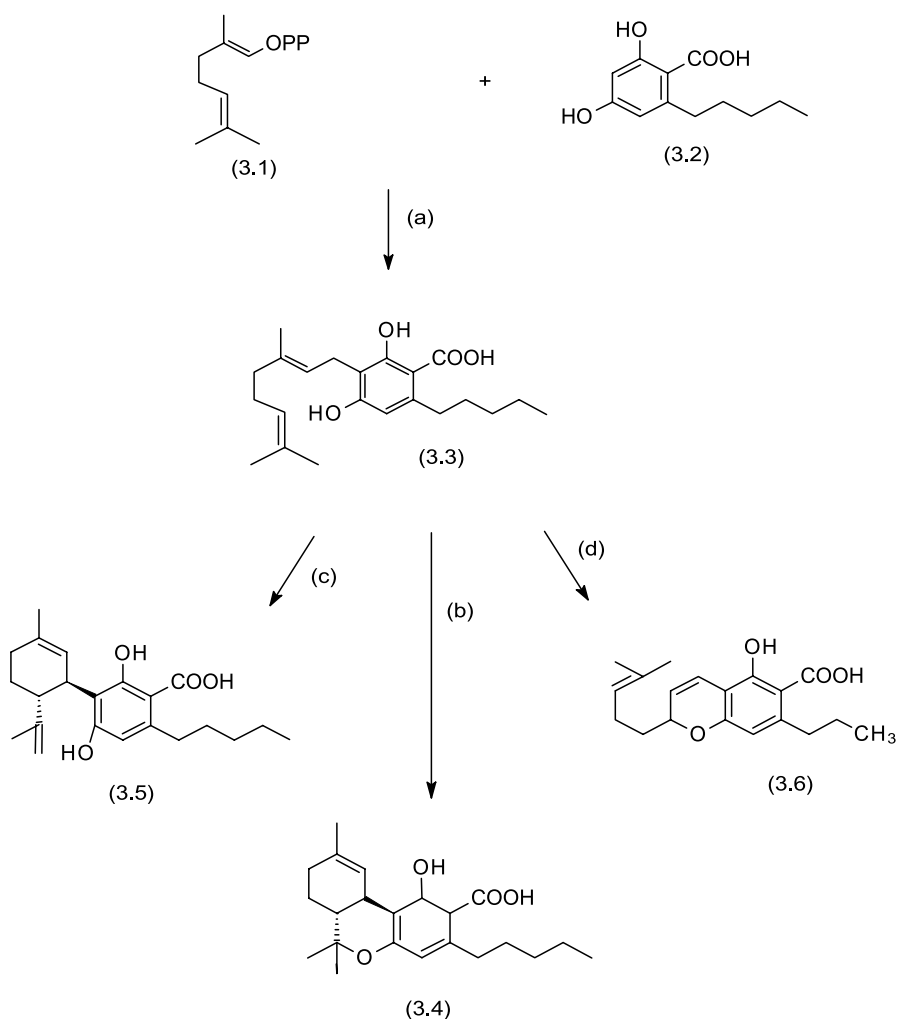


Fig. 4 Biosynthesis of THC and related cannabinoids: *a* GOT, *b* THCs, *c* CBDs, *d* CBCs

they occur as genuine compounds in the biosynthesis. Under plant physiological conditions the decarboxylated products will be absent or be present only in small amounts.

The late cannabinoid pathway starts with the alkylation of olivetolic acid (3.2 in Fig. 4) as polyketide by geranyl diphosphate (3.1) as the 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 [23, 24, 29, 30]. The mevalonate pathway is located in the cytoplasm of the plant cells [30], whereas the DXP pathway as major pathway is located in the plastids of the plant cells [29] 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 [26], or catalyze binding of one acetyl-CoA with four malonyl-CoA units [28] to biosynthesize olivetolic acid [26–28, 31, 32]. Olivetolic acid as precursor for Δ^9 -THC contains a pentyl chain in position C-3 of its phenolic system, but shorter chain lengths have also been observed in cannabinoids [33]. These differences in chain length support the hypothesis of production by a polyketide, as it is a known feature of these enzymes [34]. It was recently described that crude plant cell extracts from *C. sativa* are able to convert polyketide precursors into olivetol [26]; however here no olivetolic acid was detected. On the contrary, Fellermeier et al. [32] showed that only olivetolic acid and not olivetol could serve in the enzymatic prenylation with GPP or NPP. An older article described that both olivetol as olivetolic acid can be incorporated. Here the incorporation of radioactive labeled olivetol has been detected in very low amounts and olivetolic acid in high amounts. These reactions were performed in planta, whereas the previous reactions were performed in vitro [35]. It still remains unclear which structure, olivetol or olivetolic acid, is really preferred. Horper [36] and later Raharjo [26] 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 cannot be found in *Cannabis* extracts [35].

Until recently no enzymes able to produce olivetol-like compounds have been isolated. In an article by Funa et al., polyketide III enzymes were responsible for the formation of phenolic lipid compound [34], a natural product group that olivetol belongs to. Although the biosynthesized compounds contained a longer chain, which increased over time, the study supported 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, 3.3) [32, 35]. The formation of this compound is catalyzed by an enzyme from the group of geranyltransferases [28, 32]. This enzyme was studied in crude extracts made from young expanding leaves, where 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 [37] 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. 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 about polyketide synthase (see Table 2), the mechanism of activity remains uncertain. This means more studies must be performed to obtain the gene sequence.

The last enzymatic step of the cannabinoid pathway is the production of THCA (3.5), CBDA (3.4) or CBCA (3.6). The compounds are produced by three different enzymes. The first enzyme produces the major psychoactive compound of cannabis, THCA [21, 38]; the second and third are responsible for the production of CBDA [39] and CBCA [40], respectively. All of these enzymes belong to the enzyme group oxidoreductases [38–41], which means that 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 heterologous organisms. When cloned in a host organism, the highest activity was mostly seen in the media. Here the only exception was the introduction of the gene into hairy root cultures made from tobacco [42]. Studies performed on the enzyme sequence indicated that it contained a signal sequence upstream 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 where it is produced. Later studies proved that the enzyme is localized in the storage cavity of the glandular trichomes [21]. In the first publication it was determined that no cofactor is used by the enzyme [41], 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 cofactor.

CBDA synthase is thought to be an allozyme of THCA synthase and shows 87.9% identity on a nucleotide sequence level. Although the sequence of this

Table 2 Properties of enzymes found in cannabinoid biosynthesis

Enzyme	pH optimal /pI	Reaction rate k_{cat} (in vitro) [s^{-1}]	Localization	Metal ion	Cofactors	M_w^a [kDa]	Comments	Substrates	Refs.
GOT	7/N.R.	N.R.	?	Mg^{2+}				Olivetolic acid with GPP or NPP	[32]
CBDA	5/6.1	0.19 ^b	? ^d	None	None ^e	~75		CBGA and CBNRA	[39]
THCA	7.1/6.4	0.2 ^b -0.3 ^c	Storage cavity of glandular trichome	None	FAD ^e	~74		CBGA and CBNRA	[38, 41]
CBCA	6.5/7.1	0.04 ^b	?	None	None ^e	~71	Probably homo-dimeric	CBGA and CBNRA	[40]

^aObtained from protein isolation, not heterologously expressed

^bDetermined by purified Cannabis extract

^cDetermined by recombinant proteins isolates

^dCBDA synthase shown to carry a highly similar N-terminal signal sequence to THCA synthase. It is thus suggested that this enzyme is localized at the same position as THCA synthase. Furthermore, the precursor CBGA has been shown to be toxic for plant cells and is probably localized in the secretory cavity of the glandular trichome. This suggests that CBDA, THCA, CBCA are all localized in the storage cavity

^eActivity test with crude extract did not show the need for cofactors; however, from analysis performed on THCA synthase, it became clear that FAD is covalently bound to the enzyme. Furthermore, analysis of the enzymes THCA and CBDA showed the motif that is conserved for FAD binding

N.R. not reported

gene is known [43], there are no reports of studies where they produced and characterized it. All information gained about the enzyme was obtained using purified protein from *C. sativa* extracts [39]. 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 suggests that the CBDA is localized in the same place as the THCA synthase.

For CBCA synthase hardly any information has been published. The enzyme was 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, when it had a molecular weight of ~ 71 kDa. Furthermore, the CBCA synthase has shown to bear higher affinity for CBGA ($1717 \text{ M}^{-1}\text{S}^{-1}$) than THCA synthase and CBDA synthase (respectively $1382 \text{ M}^{-1}\text{s}^{-1}$ and $1492 \text{ M}^{-1}\text{s}^{-1}$), which is probably due to its homodimeric nature [40].

From the biosynthetic route a lot of knowledge has been gathered through the years. Up to now only one enzyme has been reasonably characterized, but much information has been gained through 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 [44] 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 for the development of new ones, as we will see in the next section.

2.2

Genetics of *Cannabis Sativa*

The majority of *C. sativa* strains exist as a dioeciously (separate sexes) plant species and are wind-pollinated. Under normal conditions it is an annual herb, although longer-living *C. sativa* have been observed [45, 46]. Some *Cannabis* strains appear as monoecious (containing both male and female parts) cultivars, such as the Ukrainian cultivar USO31 [47], 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 [48, 49]. The *C. sativa* genome is normally a diploid one and contains ten chromosome pairs ($2n = 20$). Here, eighteen are autosomal and two are sex-linked chromosomes. The genome was measured in both female (XX) as well as in male plants (XY). In contrast to animals, the male genome was found to be bigger by 47 Mbp [50, 51]. 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* plants are believed to be a monotypic species [47] called *Cannabis sativa* L. with further divisions in subspecies. However, Hillig [46] 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 [46]. There is still discussion about whether or not the *C. sativa* species are monotypic or polytypic, but in most literature they are referred to as *C. sativa* with further division into the subspecies *indica* or *ruderalis*.

C. sativa is mostly divided into three major chemotypes. The chemotype boundaries are set by the ratio CBD : THC and are calculated as percentage of 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 [47], but the former theory is the most likely one [52–54]. The locus is called the 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 [54]. 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 chemotypes and molecular analysis [47]. In crossings made with fiber-type and drug-type, the 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. [47] proved, with the help of multiplex PCR, that 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 or 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, the fiber-type group that is shown to be homogeneous for the B_d allele, still produces low amounts of THCA. It is thus still possible that the homogeneous type carries the THCA synthase gene; however, it is not detected due to the polymorphisms within the gene, as shown by Kojoma et al. [44].

Recently it has been suggested that there are two more chemotypes. The first (chemotype 4) has a high content of CBGA (B₀ allele) and the second is a strain totally lacking cannabinoids [47]. These strains are of interest because they can serve as good and safe strains for the production of fiber.

2.3

Environmental Factors

Cannabis seems to react to 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 been shown to induce sex change in *C. sativa*. Moreover, 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 a strain is indeed a fiber strain or if it is an intermediate strain that has been 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.

2.3.1

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 [55].

2.3.2

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, no profound research results have yet been published on the most optimal soil conditions.

2.3.3

Light

Light has a major influence on plants, 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 in UV-B dose [56–58].

2.4

Growing of *Cannabis Sativa* and Optimization of THC Yield

2.4.1

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 [12] and several Western countries have 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. In this chapter we discuss basic aspects of the cultivation of *C. sativa* and the optimization of THC content in the plant.

2.4.2

Optimization 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 scale or by illegal drug cultivators. In the previous section we have already discussed the fact that cannabinoid production is mostly genetically determined. This knowledge could thus be used to increase the production of certain compounds and decrease the others. 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 amounts of CBDA whereas others have none. Furthermore, some plants have been shown to contain detectable amounts of CBGA while others had none. There is still a question as to what extent THCA production can be increased 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 dependent 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 cannot 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 Sect. 2.1). Here we could think of increasing glandular trichoma densities, increasing precursor production, increasing 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, e.g., while one has the ability to grow over a few meters high, another stays small. Furthermore, variations in glandular trichoma densities have also been observed in THCA content and ratios. 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. [54] have already created a high CBGA-producing plant and in the drug culture the same has been reached for THCA [59]. In the latter, preparations were found containing more than 20% THC, while in the literature and exported Cannabis the normal values lie at 6–10%.

2.4.3

Cannabis Standardization

Just like all herbal medicinal preparations, *C. sativa* should be standardized if extracts or whole plant material are to be used for medicinal purposes. Basic requirements are that all detectable constituents 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 therefore indoors, by the company Bedrocan. 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 has been established fulfilling all criteria as a herb for medicinal use.

2.5

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 biosyn-

thetic route towards cannabinoid production it is now possible to develop biological production systems as an alternative to chemical synthesis. The major advantage of biological systems is not only 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 realize them in the near future.

2.5.1

Cell Cultures

In the literature several reports can be found on the growth of callus and cell suspension cultures [60–62]. Most of them document that no cannabinoids can be found within these cultures. Although one article by Heitrich and Binder [60] 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 [61]. To obtain cell suspensions from the callus, the same media is used as for callus growth, with the exception of agar as solidifier. In the literature, the cell suspensions made from *C. sativa* callus are mostly used for bioconversion studies. There is one report that described the use of cannabinoid precursors to determine if cannabinoid production can be induced by feeding with specific biosynthetic precursors [60]. 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. These compounds induce the apoptotic response [21]. Thus, at high levels of cannabinoid content, techniques have to be developed to extract them from the growth media for continuous production.

2.5.2

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 for maize, tobacco, potato, and rice. The main purpose is to increase their resistance toward diseases [63]. Some plants also get newly introduced products, such as vitamins [64]. Another purpose of transgenic plants is their use for production of vaccines; for instance hepatitis B vaccine

in potato plants [65]. The examples shown here are a selection of many to show the possible transgenic plants uses.

2.5.3

Heterologous Expression of Cannabinoid Biosynthetic Genes

Until now there have only been 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 [38, 42]. In the literature, the use of heterologous expression of plant metabolic enzymes have been shown to be useful in the production of several compounds [25]. 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. To date, no publications discuss the efforts of using the heterologous production of cannabinoids. This strategy could be, however, of high interest to pharmaceutical companies when some cannabinoids are approved for medicinal use.

3

Chemical Synthesis

3.1

Synthesis Routes for Δ^9 -THC

After identification of Δ^9 -THC as the major active compound in *Cannabis* and its structural elucidation by Mechoulam and Gaoni in 1964 [66], a lot of work was invested in chemical synthesis of this substance. Analogous to the biosynthesis of cannabinoids, the central step in most of the Δ^9 -THC synthesis 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 [67], verbenol [68], or chrysanthenol [69]. The employment of optically pure precursors is inevitable to get the desired (-)-*trans*- Δ^9 -THC.

A general problem during the syntheses of Δ^9 -THC is the formation of the thermodynamically more stable Δ^8 -THC, which reduces the yield of Δ^9 -THC. It is formed from Δ^9 -THC by isomerization under acidic conditions. While the usage of strong acids such as *p*-TSA or TFA leads mainly to Δ^8 -THC, the yield of Δ^9 -THC can be increased by employment of weak acids, e.g., oxalic acid [70].

Recently the most employed method for the production of Δ^9 -THC on industrial scale is the condensation of (+)-*p*-mentha-2,8-dien-1-ol (5.1 in

Fig. 5) with olivetol (5.2) in the presence of boron trifluoride etherate, $\text{BF}_3 \cdot \text{OC}(\text{C}_2\text{H}_5)_2$ with CBD as a key intermediate. This one-step synthesis of Δ^9 -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. [71] and is shown in Fig. 5

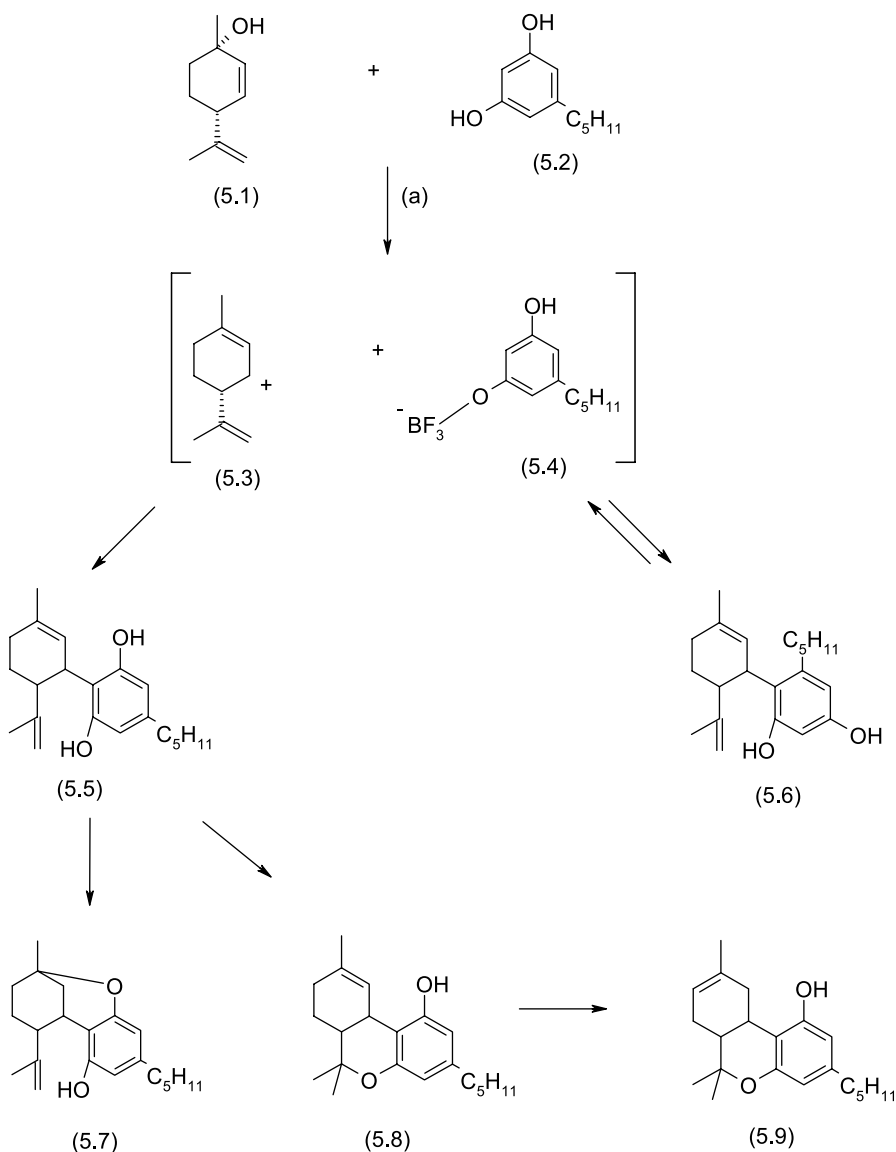


Fig. 5 Commonly used synthesis of Δ^9 -THC (a) $\text{BF}_3 \cdot \text{O}(\text{C}_2\text{H}_5)_2/\text{DCM}/\text{Mg}_2\text{SO}_4$

with the most important side products. There are two possibilities for the condensation of the active terpenoid moiety (5.3) with activated olivetol (5.4). The fusion of these compounds leads to two intermediates, normal CBD (5.5), which has the same structure as natural CBD, and “abnormal” CBD (5.6) with transposed positions of the pentyl side chain and a hydroxy group. Fortunately, the latter compound is less stable than the normal CBD and decompensates more easily. The normal CBD directly undergoes a further cyclization to Δ 9-THC (5.7). If the double bond in the terpenoid ring is used for the cyclization, a isomeric compound named *iso*-tetrahydrocannabinol (*iso*-THC, 5.8) will be formed. The reaction has to be stopped here otherwise the stable isomer Δ 8-THC (5.9) arises by decreasing the yield of Δ 9-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 distillation procedures.

3.2

Synthesis of Δ 9-Tetrahydrocannabinol from Natural Cannabidiol (Semisynthetic Δ 9-THC)

As discussed, the cultivation of *C. sativa* with high content of Δ 9-THC (drug-type) is not allowed in many countries. Because of this, there is no opportunity to harvest a high amount of the medicinally important substance Δ 9-THC directly from plant material. In the synthesis route for semisynthetic Δ 9-THC, natural CBD from fiber hemp plants is employed. It can be extracted with non-polar solvents such as petroleum ether and purified by recrystallization in *n*-pentane. This procedure avoids the formation of “abnormal” CBD and gives the opportunity to produce Δ 9-THC from fiber hemp. Semisynthetic Δ 9-THC is distinguishable from the synthetic compound because it contains, besides the major product, small amounts of Δ 9-THC-C3 and Δ 9-THC-C4, which are not available in the synthetic product.

3.2.1

Derivates of Δ 9-THC

Most relevant for the affinity for Δ 9-THC and analogs 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, many 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 positions C-11 and C-9. One of the most popular analogous compounds of Δ 9-THC is HU-210 or (–)-*trans*-11-OH- Δ 8-THC-DMH, a cannabinoid with a 1',1-dimethylheptyl side

chain (8.1). It was constructed in consideration of SAR and has a potency that is about 100 times higher than that of Δ^9 -THC itself, while its enantiomer HU-211 (Dexanabinol, 8.2) does not show this property [8]. In the synthesis of HU-210, 5-(1,1-dimethylheptyl)-resorcin is merged with modified [1S,5R]-myrtenol [72].

Nabilone (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- α -acetoglutarate as "terpenoid" module in the synthesis of Δ^9 -THC gives nabilone as an intermediate [73]. In spite of the fact that this synthesis was developed for the forming of Δ^9 -THC it also could be used for the synthesis of nabilone. A newer synthesis route is described by Archer et al. [74]. 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 Δ^9 -THC, e.g., 11-nor-9-carboxy- Δ^9 -THC (THC-COOH) [75].

Direct oxidation of Δ^9 -THC at position C-11 involves mainly an isomerization to Δ^8 -THC; another opportunity in the synthesis of Δ^9 -THC-metabolites is the pretreatment of terpenoid synthons by introduction of protective groups, e.g., 1,3-dithiane (6.1 in Fig. 6) followed by the condensation with olivetol (6.2) [76]. The formed product is a protected derivate

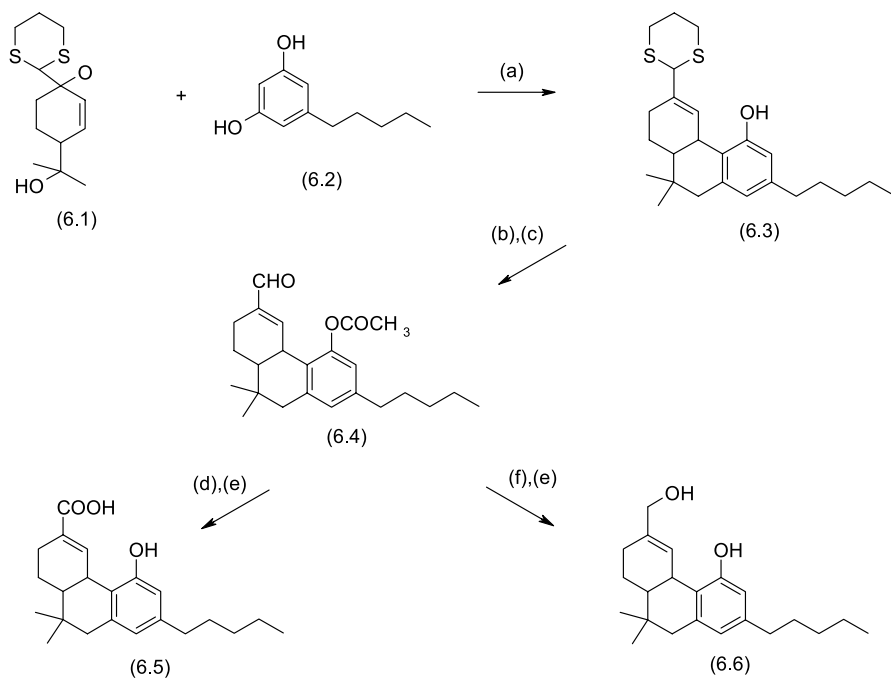


Fig. 6 Synthesis of main metabolites of Δ^9 -THC: *a* $\text{CH}_3\text{SO}_3\text{H}$, *b* $(\text{C}_2\text{H}_5\text{O})_2\text{O}$ /pyridine, *c* $\text{HgO}/\text{BF}_3 \cdot \text{O}(\text{C}_2\text{H}_5)_2$, *d* $\text{NaCN}/\text{CH}_3\text{COOH}/\text{MnO}_2$, *e* NaOH/THF , *f* $\text{NaBH}_4/\text{EtOH}$

of Δ^9 -THC (6.3), which will be modified further. 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 (6.4) can be further oxidized. Deprotection of the phenolic group by alkalic hydrolysis gives the 11-nor-9-carboxy- Δ^9 -THC (THC-COOH, 6.5). Under reductive conditions (NaBH_4 or LiAlH_4) the corresponding alcohol is formed from the aldehyde. This leads to 11-OH-THC (6.6), which is the first major metabolite from Δ^9 -THC formed in humans [77].

When [^2H]-labeled precursors are employed the resulting compounds can be used as internal standards for analysis, especially by utilization of mass spectrometric methods. Appropriate deuterated standards are shown in Fig. 7. The introduction of deuterium into the Δ^9 -THC precursors can be done with Grignard reagents such as $\text{C}[^2\text{H}_3]\text{MgI}$ or reducing substances such as $\text{LiAl}[^2\text{H}_4]$. The general procedures for the synthesis with these [^2H]-labeled precursors are the same as described above for the unlabeled compounds [76, 78].

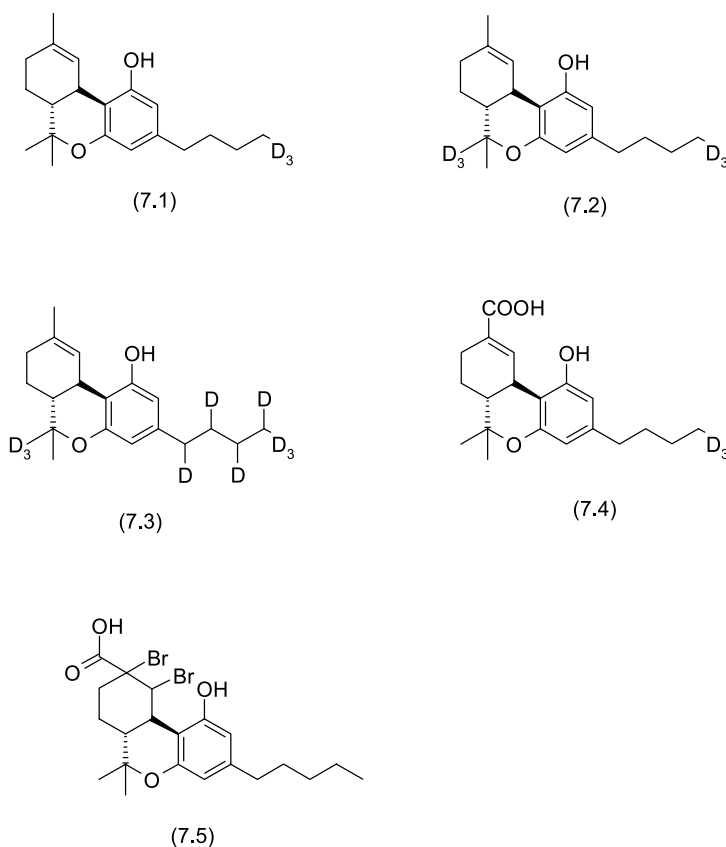


Fig. 7 Deuterated and brominated cannabinoids as internal analytical standards

While the compounds described above contain fundamentally the cannabinoidic structure, there are also compounds with radical changes but which still show 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 (8.4 in Fig. 8). A compound with total loss of the heterocyclic ring is CP-55,940 (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 [79]. Another group of multicore chemical compounds based on the indol structure as a central module in these molecules also shows affinity

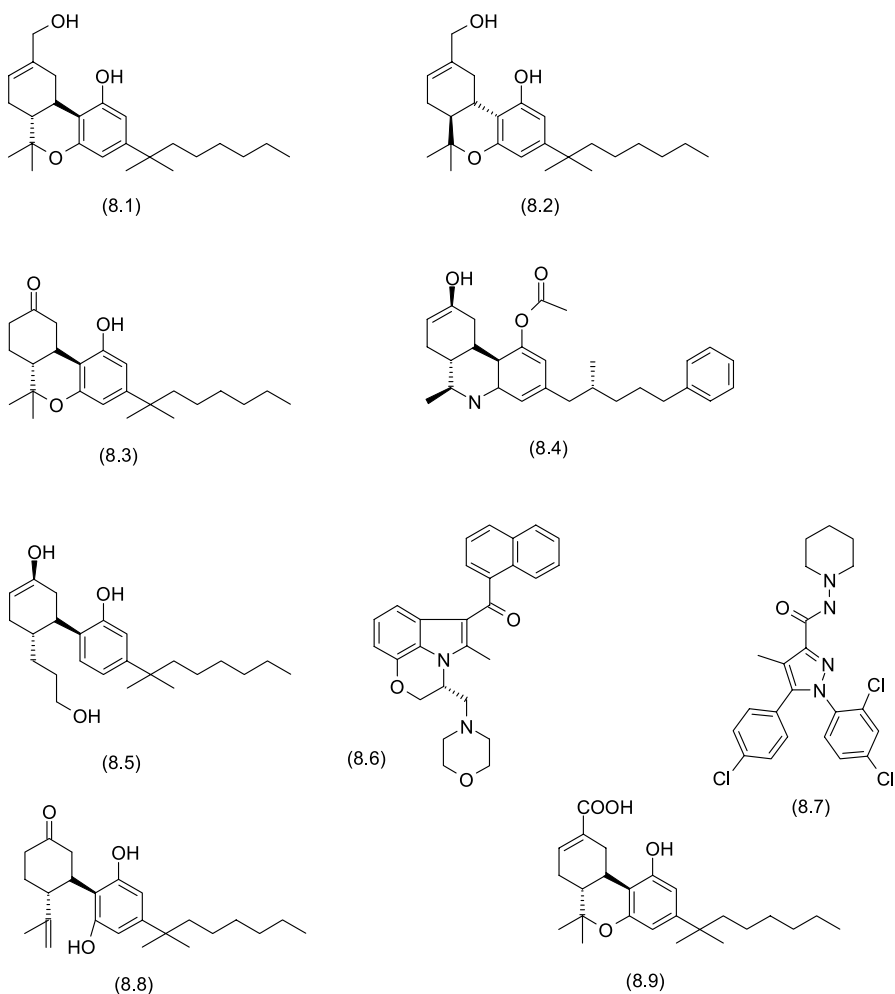


Fig. 8 Synthetic derivatives of Δ^9 -THC

to CB-receptors. The prototype of this class of aminoalkylindole cannabinoids is the substance named WIN-55,212-2 (8.6), which is quite similar to pravadoline, an anti-inflammatory drug [80].

4 Analytics

4.1 Detection of Cannabinoids in Plant Material

The chemical composition of *C. sativa* is very complex and about 500 compounds in this plant are known. A complete list can be found in [81] with some additional supplementations [2, 82]. 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 [83]. It is a widespread error that dogs that are addicted to drugs are employed for drug detection. Δ^9 -THC is an odorless substance and cannot 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 Δ^9 -THC and CBN can be used for the determination of the age of stored marijuana samples [84].

4.1.1 Analytical Methods for Detection of Δ^9 -THC and Other Cannabinoids in Plants

Many methods for determination of cannabinoids in plant material have been 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 section.

4.1.1.1 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 [85], *n*-hexane [86], petroleum ether or solvent mixtures such as methanol/chloroform [87]. The use of a second liquid-liquid extraction (LLE) with 0.1 M 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 [86]. 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 [88]. The extracts are commonly used directly for analysis. For analysis of acidic cannabinoids, as they normally appear in plant material, using GC-based methods a previous derivatization of the analytes is usually necessary.

4.1.1.2

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 that are used in GC cause the decarboxylation of acidic cannabinoids. To detect an acidic cannabinoid such as THCA together with its neutral form such as Δ^9 -THC, a derivatization is required. This procedure increases the stability of the compounds whereas their volatility is maintained. The most often used reagents for derivatization of cannabinoids in herbal samples are compounds that introduce trimethylsilyl groups (TMS) into the analytes, for example *N,O*-bis(trimethylsilyl)trifluoroacetamide (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 derivatization [89]. While the employment of established detectors such as the flame ionization detector (FID) or electron capture detector (ECD) can only give information about the quantity of a compound, the usage of mass spectrometry (MS) provides additional information about the structures of detected compounds because of their characteristic fragmentation. For the quantification of cannabinoids three-, six-, or even tenfold deuterated compounds such as shown in 7.1, 7.2 and 7.3 (Fig. 7) are often used as internal standards. The fragmentation of cannabinoids in mass spectrometry is extensively explained by Harvey and the interested reader can find more information about this topic in [90]. A table of about 50 cannabinoids containing free, derivated, and deuterated compounds with their typical mass fragmentations has been published by Raharjo and Verpoorte [89].

4.1.1.3

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 the stationary phase. Mostly the octadecyl-type

(C-18) is employed. Furthermore, the employment of a guard cartridge containing the same material as used as for the stationary phase is normally recommended. 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 (Δ^9 -THC, CBD and CBN) an isocratic method is sufficient; the separation of all cannabinoids makes a gradient elution necessary [87]. The use of a photodiode array detector (PDA) is recommended for identification of herbal cannabinoids because of their characteristic UV spectra. If a PDA is used for the detection of cannabinoids Δ^8 -THC can be employed as an internal standard [91]. 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 [92]. As described in the section on 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 use of a UV detector lacks this sensitivity. Another possibility structural identification gives the coupling of HPLC with NMR. The interpretation of [^1H]-signals that are specific for different substances can also be used for quantification [93].

4.1.1.4

Immunologically Based Techniques

The enzyme-linked immunosorbent 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 that bind with high affinity to certain molecular structures. Testing of cannabinoids by antibodies has been under investigation since the 1970s. The first detections were performed with radiolabeled antibodies made by injection of conjugates from THC, its hemisuccinate, and bovine serum albumin [94]. 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 for cannabinoids and related metabolites [95]. Furthermore, they tested against human cannabinoid metabolites excreted via urine and showed that the antibodies against plant cannabinoids were also highly selective and did not bind to any of the non-cannabinoid phenolics. In the early days these studies were all performed with polyclonal antibodies, later monoclonal antibodies were tested and documented the same results [96, 97]. These antibodies may also be used for research. For instance, labeled antibodies have been used against the THC structures to show 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 [19]. This indicates that detection by antibodies has an added value over other detection methods such as HPLC

and GC. It is thus possible to use these tests either with enzyme, fluorescent or radioactive labels to detect cannabinoids and their metabolites.

4.1.1.5 Molecular Markers and PCR

These detection mechanisms are not able to detect the small organic structure of the cannabinoids. 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 in Sect. 2.2) was possible. Within the groups selected PCR allowed 100% identification of the chemotypes without any cross reactivity [47]. Furthermore, by a simple PCR technique (two primers used) a separation could be made between drug-type and fiber-type plants [44]. However, it must be stated that a very small number of plants was used and even then polymorphism on the THCA synthase gene was found. The PCR technique cannot 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 easier.

4.2 Detection of Δ 9-THC and its Human Metabolites in Forensic Samples

Δ 9-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 Δ 9-THC-containing drugs by individuals. A careful interpretation of the results is very important to avoid fallacies with regard to the behavior of individuals. The *Cannabis* influence factor (CIF), for example, is an useful tool for distinguishing between acute and chronic intake of Δ 9-THC [98].

4.2.1 Metabolism of Δ 9-THC by Humane Cytochrome P450 Enzymes

Like other xenobiotics, cannabinoids also undergo extensive metabolism in the human body to increase their hydrophilic properties for a facilitated elimination. The metabolism of Δ 9-THC has been very well investigated. More than 100 metabolites of Δ 9-THC are known [99] and a good overview of the most important human metabolites is given in [100]. Metabolism takes place mainly in hepatic microsomes, but also in intestines, brain,

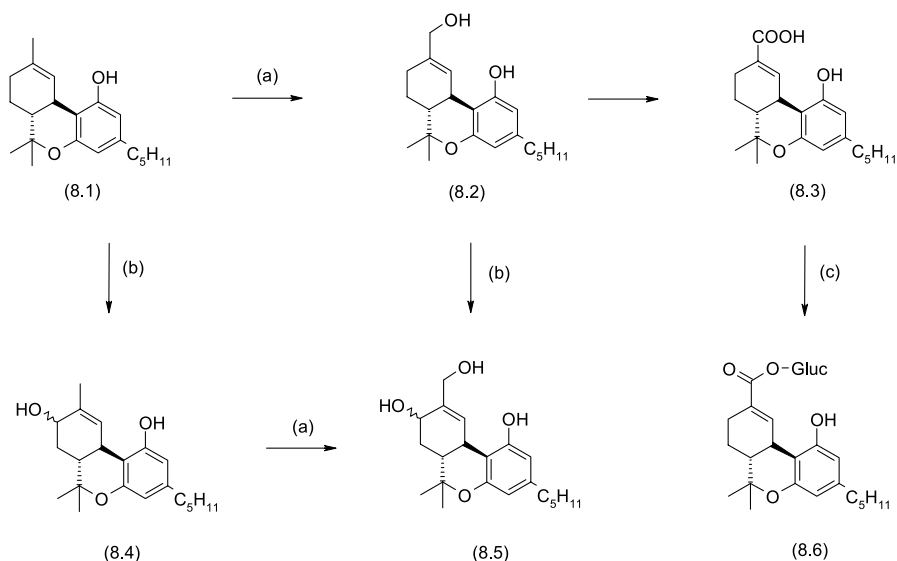


Fig. 9 Main metabolic pathways of Δ^9 -THC in humans: *a* CYP 2C9, *b* CYP 3A4, *c* UGT

heart, lung, and nearly all tissues of the body. Main metabolites of Δ^9 -THC are mono-, di- and trihydroxylated compounds, which become carboxylated and glucuronidated further. The metabolism pathway of Δ^9 -THC and its most important metabolites are shown in Fig. 9. Mostly responsible for metabolism of Δ^9 -THC in the primary pathway in humans is the cytochrome P450 isoenzyme CYP 2C9 [101]. Hydroxylation of Δ^9 -THC (9.1) at C-11 leads to 11-hydroxy- Δ^9 -THC (11-OH-THC, 9.2), which undergoes further oxidation to 11-nor-9-carboxy- Δ^9 -THC (THC-COOH, 9.3). 11-OH-THC shows similar psychotropic properties to Δ^9 -THC whereas THC-COOH is a non-psychotropic compound [102]. CYP 3A4 is the second major cytochrome P450 isoenzyme that is involved in metabolism of Δ^9 -THC – mainly with hydroxylation at C-8 to 8-OH- Δ^9 -THC, (9.4) [101]. The epoxidation of Δ^9 -THC at C-9 and C-10 is also described, in addition to oxidation of the alkyl side chain and a following cleavage [8]. Monohydroxylated Δ^9 -THC can be hydroxylated again, which leads to 8,11-dihydroxy- Δ^9 -THC, (9.5), for example. Metabolites that are formed by CYP 3A4 represent a minority in comparison to those of CYP 2C9. The glucuronide of THC-COOH, (9.6), which is formed in the secondary pathway is a human metabolite of Δ^9 -THC.

4.2.2

Analytical Methods for Detection of Δ^9 -THC and its Metabolites

As described for the analysis of the plant, GC, HPLC, and immunoassays are commonly used for the analysis of body fluids. Although the general proced-

ures are quite similar to those used in the analysis of *C. sativa* (see Sect. 4.1.1) some differences must be pointed out.

4.2.2.1

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 [103] is recommended.

Pretreatment of hair samples also includes an extraction, usually with an alkaline 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 [104–106]. In the 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 to extraction [107]. Storage of collected samples is another important fact that can cause false results in their content of Δ^9 -THC and metabolites [108–110].

4.2.2.2

Gas Chromatographic Methods (GC)

The preferred detection method for cannabinoids in forensic samples is GC-MS with or without preceding derivatization. As described before in the analysis of plant materials, the employment of silica-fused columns is recommended in the analysis of human body fluids. While in analysis of *Cannabis* TMS-reagents are mostly employed for derivatization, in the case of human body material fluoric compounds such as pentafluoropropionic anhydride (PFPA) or 2,2,3,3,3-pentafluoro-1-propanol (PFPOH) as derivatization reagents are used [89]. Halogenation of the analytes in these ways allows the use of an electron capture detector (ECD) to find the desired compounds. In comparison with other detectors such as the flame ionization detector (FID), the detection sensitivity 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, acylation reagents such as trifluoroacetic anhydride (TFAA) and *N*-methyl-bis(trifluoroacetamide) (MBTFA) are also used for analysis of cannabinoids in human materials [111–114]. Trideuterated THC-COOH (**7.4**) is the most commonly used internal standard for the analysis of metabolites

with GC-MS. Baptista et al. [103] have shown that the limit of quantification (LOQ) for the important metabolite THC-COOH is much more better if negative chemical ionization (NCI) is used instead of electron ionization (EI).

4.2.2.3

Liquid Chromatographic Methods (HPLC)

Whilst for the analysis of plant material for cannabinoids both GC and HPLC are commonly used, in analytical procedures the employment of GC-based methods prevails for human forensic samples. Nonetheless, the usage of HPLC becomes more and more of interest in this field especially in combination with MS [115–120]. Besides the usage of deuterated samples as internal standards Fisher et al. [121] describe the use of a dibrominated THC-COOH (see 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 rather than GC could be the integration of SPE cartridges, which are needed for sample preparation in the HPLC-system.

4.2.2.4

Immunoassays

Most of the tests that were developed for detection of cannabinoids in plants have shown that antibodies are specific for the cannabinoid structure. Because of this specificity these tests can be extensively applied for the detection of cannabinoids and metabolites in human body fluids such as plasma, urine, and oral fluids. Many different kits based on these methods were developed and they are commercially available, for example Oratect, Branan or Up-link, and OraSure. We must consider, however, that no humans have the same metabolite profile in their blood and that cross-reactivity may always occur [122, 123]. Nevertheless, these tests offer a simple way of excluding most of the suspicious samples, but the results still have to be confirmed with a second method such as GC-MS [124, 125].

5

Medicinal use of *Cannabis* and Cannabinoids

5.1

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 [126]. Modern medicinal use was mainly introduced by William B. O'Shaughnessy who

was one of the first physicians who systematically explored its therapeutic potential [127]. Studying the literature of the 19th century it is impressive how efficiently most indications, which are now under intensive research, were already depicted by observation and simple trial and error.

5.2

Modern Use

5.2.1

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 particularities due to the structure of the active ingredients of *C. sativa*. The identity of the main active constituent of *C. sativa*, Δ^9 -tetrahydrocannabinol (INN dronabinol) remained unknown until 1964 [128]; standardized *C. sativa* preparations were not available. The plant itself is found in several different chemotypes, which added to the unpredictable nature of early medicinal preparations.

Cannabinoids are highly lipophilic compounds making bioavailability very dependent on the formulation and the mode of administration. Cannabinoid occurrence in the plant is predominantly in the form of the carboxylic acids, which are pharmacologically totally different and rather unstable, decarboxylating over 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.

Last but not least the identification of THC as the main active constituent of *C. sativa* was preceded by an almost total ban on 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 purposes. 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 Δ^9 -THC was established and it became the first cannabinoid available as a modern medicine in the form of soft gel capsules (the active ingredient being called dronabinol from tetrahydrocannabinol) 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 marijuana-smoking patients, not from pharmacological research [129].

The second indication, being licensed for THC several years later, came from an observation that had been known for a long time for *C. sativa*, namely its appetite-stimulating effects. This sometimes very impressive effect (popularly 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 [130].

Pharmacological research and the non-prescriptional use of *C. sativa* by patients gave way to new indications. Now well established are the efficacies for the following indications:

- Nausea and vomiting [129]
- Appetite stimulation [131, 132]
- Spasticity [133, 134]
- Tourette syndrome [135]
- Neuropathic pain [136]
- Multiple sclerosis [137]
- Mood elevation
- Glaucoma [138]
- 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 the mechanisms for the already known effects were clarified. 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 the focus of medicinal research.

CBD was first isolated from *C. sativa* in 1940 [139]. Unlike the resinous air-sensitive THC, CBD is a crystalline stable substance. Its 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 co-administered [140]. Another effect of CBD is the inhibition of cytochrome oxidase [141], which inversely to its antagonistic activity strongly potentiates THC effects above a certain threshold. CBD is also active as a mild antipsychotic [142] 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 endocannabi-

noid system. It is now also under research for the treatment of diabetes and obesity [143].

5.2.2

Synthetic Cannabinoids

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

5.2.2.1

Nabilone

In contrast to THC (an oxygen-sensitive resin), nabilone (**8.3**) is a crystalline stable substance. It is about five to ten times more potent than THC [144]. 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.

5.2.2.2

Levonantradol

Levonantradol (**8.4**) was synthesized with the intention to introduce a basic amino function into the heterocycle in the hope of obtaining water-soluble salts. Although the solubility of the hydrochloride is not good it was possible to get stable aqueous micellar solutions with the aid of emulsifiers [145] and the compound made its way as an injectable into clinical trials, but never was approved.

5.2.2.3

CP-55,940

CP-55,940 (**8.5**) was developed during the search for novel analgesics [146]. Although it is more potent than morphine it was never approved. Nevertheless, in its tritium-labeled form it became a very important tool for research and helped in the first identification of the cannabinoid receptor.

5.2.2.4

WIN-55,212-2

In the search for new anti-inflammatory drugs structurally derived from indomethacine [147], 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 (**8.6**), which has a higher affinity to the CB1 receptor than THC [148]

and became an important research tool. The side effects of substances possessing agonistic activity on the CB1 receptor (mainly psychotropic effects similar to those of cannabis) limited its clinical use and changed the focus of research to the development of compounds without this drawback.

5.2.2.5

Rimonabant

Rimonabant or SR-141716A (**8.7**) is an antagonist at the CB1 receptor [149] 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 homolog of THC) also acts as an antagonist on the CB1 receptor and might become a competitor for rimonabant.

5.2.2.6

PRS-211,096

PRS-211,096 (**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.

5.2.2.7

HU-211

HU-210 is (**8.1**) among the most potent cannabinoids known. Its enantiomer HU-211 (**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.

5.2.2.8

Ajulemic Acid

Ajulemic acid (CT3, **8.9**) is the dimethylheptyl homolog of the main metabolite of Δ^8 -THC. It has no psychotropic activity, but has analgesic and anti-inflammatory effects.

5.3

Drug Delivery

The classical way of application of Δ^9 -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 various diseases as discussed above use this form of unprescribed self-medication.

Besides the inhalative use, the development of a drug formulation for $\Delta 9$ -THC has to address other bioavailability questions. A major problem is the lipophilicity and poor solubility in water, limiting oral uptake when given orally. Because of this, other parenteral routes of application are under investigation like pulmonary uptake by vaporization, sublingual or intranasal administration, and application by injection of $\Delta 9$ -THC incorporated in liposomes.

Marinol and Sativex are given orally to the patient but, as indicated, the poor solubility of $\Delta 9$ -THC is responsible for its slow onset and release from drug carriers like soft gelatine capsules [150]. 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 $\Delta 9$ -THC derivatives with improved solubility (e.g., dexanabinol, which is a hemisuccinate prodrug). However, this strategy is mostly not desirable because of the high risk involved in the cost and time-consuming drug approval process to gain all toxicological and clinical data.

The main strategies in pharmaceutical technology to improve solubility are the reduction of particle size and the increase of particle surface according to the Kelvin equation. These two strategies have been applied for $\Delta 9$ -THC production by solid dispersion technology and production of nanosuspensions. Van Drooge et al., created a solid dispersion of inulin in which $\Delta 9$ -THC was incorporated [151]. Applying freeze drying techniques for evaporation of a mixture of water and tertiary butyl alcohol, which acts as dissolving medium for $\Delta 9$ -THC and inulin, forms amorphous $\Delta 9$ -THC in a fast-dissolving solid inulin matrix. The main advantage of the technique is to protect $\Delta 9$ -THC from degeneration and to optimize the dissolution rate from tablets [151]. A second and easy way to increase the solubility can be achieved by reduction of the particle size. In unpublished work by the author's group, nanosuspensions of $\Delta 9$ -THC have been achieved indicating a first significant improvement on the physical properties. The main drawbacks of the technique is the poor stability of the highly energetic suspensions and the risk of forming cluster and microparticles without sufficient stabilization of the nanosuspension. Perlin et al., applied $\Delta 9$ -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 [152]. The authors concluded that intramuscularly injection is favorable because of a bioavailability of $89\% \pm 16\%$ (i.m.) versus $26\% \pm 14\%$ (p.o.). Interestingly Perlin et al. mentioned that rectal administration was not successful and no significant blood levels were detected [152]. More recently Munjal et al., developed a transmucosal system based on polyethylene oxide (PEO) polymers, which are commonly used for

the production of suppositories [150]. In this study the heat-labile Δ 9-THC hemisuccinate was used to produce suppositories varying in PEO composition by the hot-melt technique (120 °C). Temperature led to a degradation of between 13.5% and 49.4% depending on the composition, but incorporation of vitamin E succinate reduced processing degradation to 9.2% and gave a shelf half-life of 8 months. No data have been published yet to characterize the bioavailability or pharmacological effect.

To achieve reliable elevated plasma levels and to overcome the first pass effect, alternative parenteral administration systems have been developed. The most obvious route is vaporization of the *Cannabis* plant material or the Δ 9-THC directly. Hazekamp et al., conducted an intensive study using the Volcano device [153]. The main principle is evaporation of Δ 9-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. The main advantage of the Volcano vaporizer is that Δ 9-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 of therapeutic blood levels. To compare with alternative smoking procedures, the Δ 9-THC recovery was 54% for the Volcano and 39% for the water pipe.

Pulmonary application can be still unpleasant for non-smokers, which is why other administration routes like sublingual or intranasal uptake are also of interest. Valiveti et al., investigated nasal application for Δ 9-THC and WIN-55,121-2 mesylate in rats [154]. The latter is a synthetic cannabinoid with a short half life time and a highly variable bioavailability. Both drugs were formulated in ethanol and propylene glycol and were successfully administered. In comparison with i.v. applied reference drugs, a tenfold higher nasal dose (10 mg/kg Δ 9-THC) showed similar AUC values with a slightly increased half-life time.

A second alternative is sublingual application, as introduced by Mannila et al., based on cyclodextrin matrices [155]. Cyclodextrins are a group of cyclic oligosaccharides that have been shown to improve aqueous solubility, dissolution rate, and bioavailability of various lipophilic drugs such as testosterone or prostaglandin E, to give two examples. Cyclodextrins 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 Δ 9-THC and cannabidiol (prepared by freeze drying) with randomly methylated β -cyclodextrin and hydroxypropyl- β -cyclodextrin (HP- β -CD) was studied by the phase-solubility method. 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 cyclodextrins increased both the aqueous solubility and dissolution rate

of these cannabinoids, making the development of novel sublingual formulation possible, which has been shown by in vivo studies in New Zealand rabbits.

References

1. Shulgin AT (1968) *J Psychedelic Drugs* 2:14
2. Elsohly MA, Slade D (2005) *Life Sci* 78:539
3. Pate DW (2004) In: Grotenhermen F (ed) *Cannabis un Cannabinoide*. Hans Huber, Bern, p 33
4. Garrett ER, Hunt CA (1974) *J Pharm Sci* 63:1056
5. Uliss DB, Handrick GR, Dalzell HC, Razdan RK (1978) *Tetrahedron* 34:1885
6. Gaoni Y, Mechoulam R (1971) *J Am Chem Soc* 93:217
7. Kriwacki RW, Makriyannis A (1989) *Mol Pharmacol* 35:495
8. Mechoulam R, Devane WA, Glaser R (1999) In: Nahas GG, Sutin KM, Harvey DJ, Agurell S (eds) *Marihuana and medicine*. Humana, New Jersey, p 65
9. Gaoni Y, Mechoulam R (1964) *J Am Chem Soc* 86:1646
10. Miller IJ, McCallum NK, Kirk CM, Peake BM (1982) *Cell Mol Life Sci (CMLS)* 38:230
11. Turner CE, Elsohly MA (1979) *J Heterocycl Chem* 16:1667
12. Ranalli P, Venturi G (2004) *Euphytica* 140:1
13. Collin C, Davies P, Mutiboko IK, Ratcliffe S (2007) *Eur J Neurol* 14:290
14. Ligresti A, Moriello AS, Starowicz K, Matias I, Pisanti S, De Petrocellis L, Laezza C, Portella G, Bifulco M, Di Marzo V (2006) *J Pharmacol Exp Ther* 318:1375
15. Williamson EM, Evans FJ (2000) *Drugs* 60:1303
16. Charles T, Hammond PGM (1973) *Am J Botany* 60:542
17. Turner JC, Hemphill JK, Mahlberg PG (1981) *Bull Narc* 33:63
18. Turner JC, Hemphill JK, Mahlberg PG (1981) *Bull Narc* 33:59
19. Eun-Soo Kim PGM (1997) *Am J Botany* 84:336
20. Jocelyn C, Turner JKH, Mahlberg PG (1978) *Am J Botany* 65:1103
21. Sirikantaramas S, Taura F, Tanaka Y, Ishikawa Y, Morimoto S, Shoyama Y (2005) *Plant Cell Physiol* 46:1578
22. Lange BM, Rujan T, Martin W, Croteau R (2000) *Proc Natl Acad Sci USA* 97:13172
23. Eisenreich W, Bacher A, Arigoni D, Rohdich F (2004) *Cell Mol Life Sci* 61:1401
24. Rohdich F, Kis K, Bacher A, Eisenreich W (2001) *Curr Opin Chem Biol* 5:535
25. Julsing MK, Koulman A, Woerdenbag HJ, Quax WJ, Kayser O (2006) *Biomol Eng* 23:265
26. Raharjo TJ, Chang W-T, Choi YH, Peltenburg-Looman AMG, Verpoorte R (2004) *Plant Sci* 166:381
27. Raharjo TJ, Chang WT, Verberne MC, Peltenburg-Looman AM, Linthorst HJ, Verpoorte R (2004) *Plant Physiol Biochem* 42:291
28. Fellermeier M, Eisenreich W, Bacher A, Zenk MH (2001) *Eur J Biochem* 268:1596
29. Lichtenthaler HK, Schwender J, Disch A, Rohmer M (1997) *FEBS Lett* 400:271
30. Lichtenthaler HK (1999) *Annu Rev Plant Physiol Plant Mol Biol* 50:47
31. Austin MB, Noel JP (2003) *Nat Prod Rep* 20:79
32. Fellermeier M, Zenk MH (1998) *FEBS Lett* 427:283
33. Shoyama Y, Hirano H, Nishioka I (1984) *Phytochemistry* 23:1909
34. Funai N, Ozawa H, Hirata A, Horinouchi S (2006) *Proc Natl Acad Sci USA* 103:6356
35. Kajima M, Piraux M (1982) *Phytochemistry* 21:67

36. Horper W, Marner F-J (1996) *Phytochemistry* 41:451
37. Kuzuyama T, Noel JP, Richard SB (2005) *Nature* 435:983
38. Sirikantaramas S, Morimoto S, Shoyama Y, Ishikawa Y, Wada Y, Shoyama Y, Taura F (2004) *J Biol Chem* 279:39767
39. Taura F, Morimoto S, Shoyama Y (1996) *J Biol Chem* 271:17411
40. Morimoto S, Komatsu K, Taura F, Shoyama Y (1998) *Phytochemistry* 49:1525
41. Taura F, Morimoto S, Shoyama Y, Mechoulam R (1995) *J Am Chem Soc* 117:9766
42. Taura U (2004) Research report, Graduate School of Pharmaceutical Sciences, Kyushu University. Available at <http://www.nisr.or.jp/englishHP/report2004/NISR04taura.pdf>, last visited: 29 June 2007
43. Yoshikai K, Morimoto S, Shoyama Y (2001) Japanese Patent 2000-78979
44. Kojoma M, Seki H, Yoshida S, Muranaka T (2006) *Forensic Sci Int* 159:132
45. Cherniak L (1982) The great books of *Cannabis*, vol I, Book II. Damele, Oakland, CA
46. Hillig KW (2005) *Gene Res Crop Evolut* 52:1573
47. Pacifico D, Miselli F, Micheler M, Carboni A, Ranalli P, Mandolino G (2006) *Mole Breed* 17:257
48. Renner SS, Ricklefs RE (1995) *Am J Bot* 82:596
49. Sakamoto K, Abe T, Matsuyama T, Yoshida S, Ohmido N, Fukui K, Satoh S (2005) *Genome* 48:931
50. Sakamoto K, Akiyama Y, Fukui K, Kamada H, Satoh S (1998) *Cytologica* 63:459
51. Mandolino GAC, Bagatta MVM, Moliterni C, Ranalli P (2002) *Euphytica* 126:211
52. Mandolino G, Bagatta M, Carboni A, Ranalli P, Meijer E (2003) *J Industrial Hemp* 8:51
53. de Meijer EPM, Bagatta M, Carboni A, Crucitti P, Moliterni VMC, Ranalli P, Mandolino G (2003) *Genetics* 163:335
54. de Meijer EPM, Hammond KM (2005) *Euphytica* 145:189
55. Sharma GK (1975) *Bull Torrey Bot Club* 102:199
56. Jansen M, Gaba V, Greenberg BM (1998) *Trends Plant Sci* 3:131
57. Lydon J (1986) Effects of ultraviolet-B radiation on the growth, physiology and cannabinoid production of *Cannabis sativa* L. Dissertation, Maryland University, USA
58. Lydon J, Teramura AH, Coffman CB (1987) *Photochem Photobiol* 46:201
59. Pijlman FTA, Rigter SM, Hoek J, Goldschmidt HJM, Niesink RJM (2005) *Addiction Biol* 10:171
60. Heitrich A, Binder M (1982) *Cell Mol Life Sci* 38:898
61. Feeney M, Punja ZK (2003) *In Vitro Cell Develop Biol Plant* 39:578
62. Braemer R, Paris M (1987) *Plant Cell Reports* 6:150
63. Koziel MG, Beland GL, Bowman C, Carozzi NB, Crenshaw R, Crossland L, Dawson J, Desai N, Hill M, Kadwell S, Launis K, Lewis K, Maddox D, McPherson K, Meghji MR, Merlin E, Rhodes R, Warren GW, Wright M, Evola SV (1993) *Nat Biotech* 11:194
64. Herbers K (2003) *J Plant Physiol* 160:821
65. Mason HS, Lam DM, Arntzen CJ (1992) *Proc Natl Acad Sci USA* 89:11745
66. Gaoni Y, Mechoulam R (1964) *J Am Chem Soc* 86:1646
67. Mechoulam R, Braun P, Gaoni Y (1972) *J Am Chem Soc* 94:6159
68. Mechoulam R, Braun P, Gaoni Y (1967) *J Am Chem Soc* 89:4552
69. Razdan RK, Handrick GR, Dalzell HC (1975) *Experientia* 31:16
70. Petrzilka T, Haefliger W, Sikemeier C (1969) *Helv Chim Acta* 52:1102
71. Razdan RK, Dalzell HC, Handrick GR (1974) *J Am Chem Soc* 96:5860
72. Mechoulam R, Lander N, University A, Zahalka J (1990) *Tetrahedron: Asymmetry* 1:315
73. Fahrenholtz KE, Lurie M, Kierstead RW (1967) *J Am Chem Soc* 89:5934

74. Archer RA, Blanchard WB, Day WA, Johnson DW, Lavagnino ER, Ryan CW, Baldwin JE (1977) *J Org Chem* 42:2277
75. Kachensky DF, Hui AHF (1997) *J Org Chem* 62:7065
76. Szirmai M (1995) Total synthesis and analysis of major human urinary metabolites of di-tetrahydrocannabinol, the principal psychoactive component of *Cannabis sativa* L. Dissertation, Uppsala University, Sweden
77. Siegel C, Gordon PM, Razdan RK (1989) *J Org Chem* 54:5428
78. Seltzman HH, Begum KM, Wyrick DC (1991) *J Labelled Compds Radiopharm* 29:1009
79. Melvin LS, Milne GM, Johnson MR, Subramaniam B, Wilken GH, Howlett AC (1993) *Mol Pharmacol* 44:1008
80. Eissenstat MA, Bell MR, D'Ambra TE, Alexander EJ, Daum SJ, Ackerman JH, Gruett MD, Kumar V, Estep KG, Olefirowicz EM, et al. (1995) *J Med Chem* 38:3094
81. Turner CE, Elsohly MA, Boeren EG (1980) *J Nat Prod* 43:169
82. Ross SA, El-Sohly MA (1995) *Zagazig J Pharm Sci* 4:1
83. Mediavilla V, Steinemann S (1997) *J Inter Hemp Assoc* 4:80
84. Ross SA, ElSohly MA (1998) *Bull Narcotics*
85. Bacigalupo MA, Ius A, Meroni G, Grassi G, Moschella A (1999) *J Agric Food Chem* 47:2743
86. Lercker G, Bocci F, Frega N, Bortolomeazzi R (1992) *Farmaco* 47:367
87. Lehmann T, Brenneisen R (1995) *J Liquid Chrom* 18:689
88. Ross SA, Mehmedic Z, Murphy TP, Elsohly MA (2000) *J Anal Toxicol* 24:715
89. Raharjo TJ, Verpoorte R (2004) *Phytochem Anal* 15:79
90. Harvey DJ (1987) *Mass Spectrom Rev* 6:135
91. Rustichelli C, Ferioli V, Baraldi M, Zanolì P, Gamberini G (1998) *Chromatographia* 48:215
92. Hazekamp A, Peltenburg A, Verpoorte R, Giroud C (2005) *J Liquid Chrom Rel Technol* 28:2361
93. Hazekamp A, Choi YH, Verpoorte R (2004) *Chem Pharm Bull* 52:718
94. Teale JD, Forman EJ, King LJ, Piall EM, Marks V (1975) *J Pharm Pharm* 27:465
95. ElSohly MA, Jones AB, ElSohly HN (1990) *J Anal Toxicol* 14:277
96. Tanaka H, Goto Y, Shoyama Y (1996) *J Immunoassay* 17:321
97. Tanaka H, Shoyama Y (1999) *Forensic Sci Int* 106:135
98. Musshoff F, Madea B (2006) *Ther Drug Monit* 28:155
99. Brenneisen R (2004) In: Grotenhermen F (ed) *Cannabis und Cannabinoide*. Hans Huber, Bern, p 89
100. Agurell S, Halldin M, Lindgren JE, Ohlsson A, Widman M, Gillespie H, Hollister L (1986) *Pharmacol Rev* 38:21
101. Watanabe K, Yamaori S, Funahashi T, Kimura T, Yamamoto I (2007) *Life Sci* 80:1415
102. Grotenhermen F (2003) *Clin Pharmacokinet* 42:327
103. Baptista MJ, Monsanto PV, Pinho Marques EG, Bermejo A, Avila S, Castanheira AM, Margalho C, Barroso M, Vieira DN (2002) *Forensic Sci Int* 128:66
104. Sporkert F, Pragst F (2000) *Forensic Sci Int* 107:129
105. Musshoff F, Lachenmeier DW, Kroener L, Madea B (2003) *Forensic Sci Int* 133:32
106. de Oliveira CDR, Yonamine M, de Moraes Moreau RL (2007) *J Sep Sci* 30:128
107. Boumba VA, Ziavrou KS, Vougiouklakis T (2006) *Int J Toxicol* 25:143
108. Skopp G, Potsch L (2004) *J Anal Toxicol* 28:35
109. Jamerson MH, McCue JJ, Klette KL (2005) *J Anal Toxicol* 29:627
110. Moore C, Vincent M, Rana S, Coulter C, Agrawal A, Soares J (2006) *Forensic Sci Int* 164:126

111. Kim JY, Suh SI, In MK, Paeng KJ, Chung BC (2005) *Arch Pharm Res* 28:1086
112. Nadulski T, Sporkert F, Schnelle M, Stadelmann AM, Roser P, Schefter T, Pragst F (2005) *J Anal Toxicol* 29:782
113. Jamerson MH, Welton RM, Morris-Kukoski CL, Klette KL (2005) *J Anal Toxicol* 29:664
114. Moore C, Rana S, Coulter C, Feyerherm F, Prest H (2006) *J Anal Toxicol* 30:171
115. Franski R, Tezyk A, Wachowiak R, Schroeder G (2004) *J Mass Spectrom* 39:458
116. Concheiro M, de Castro A, Quintela O, Cruz A, Lopez-Rivadulla M (2004) *J Chromatogr B Analyt Technol Biomed Life Sci* 810:319
117. Maralíkova B, Weinmann W (2004) *J Mass Spectrom* 39:526
118. Teixeira H, Proenca P, Verstraete A, Corte-Real F, Vieira DN (2005) *Forensic Sci Int* 150:205
119. Kolmonen M, Leinonen A, Pelander A, Ojanpera I (2007) *Anal Chim Acta* 585:94
120. Laloup M, Ramirez Fernandez MM, Wood M, De Boeck G, Henquet C, Maes V, Samyn N (2005) *J Chromatogr A* 1082:15
121. Fisher DH, Broudy MI, Fisher LM (1996) *Biomed Chromatogr* 10:161
122. Walsh JM, Crouch DJ, Danaceau JP, Cangianelli L, Liddicoat L, Adkins R (2007) *J Anal Toxicol* 31:44
123. Clarke J, Wilson JF (2005) *Forensic Sci Int* 150:161
124. Moody DE, Fang WB, Andrenyak DM, Monti KM, Jones C (2006) *J Anal Toxicol* 30:50
125. Cirimele V, Villain M, Mura P, Bernard M, Kintz P (2006) *Forensic Sci Int* 161:180
126. Li HL (1974) *Econ Bot* 28:437
127. O'Shaughnessy WB (1838) *Trans Med Phys Soc Bengal*, p 71
128. Gaoni Y, Mechoulam R (1964) *J Am Chem Soc* 86:1646
129. Sallan SE, Zinberg NE, Frei E 3rd (1975) *N Engl J Med* 293:795
130. Palenicek JP, Graham NM, He YD, Hoover DA, Oishi JS, Kingsley L, Saah AJ (1995) *J Acquir Immune Defic Syndr Hum Retrovirol* 10:366
131. Beal JE, Olson R, Laubenstein L, Morales JO, Bellman P, Yangco B, Lefkowitz L, Plasse TF, Shepard KV (1995) *J Pain Symptom Manage* 10:89
132. Beal JE, Olson R, Lefkowitz L, Laubenstein L, Bellman P, Yangco B, Morales JO, Murphy R, Powderly W, Plasse TF, Mosdell KW, Shepard KV (1997) *J Pain Symptom Manage* 14:7
133. Brenneisen R, Egli A, Elsohly MA, Henn V, Spiess Y (1996) *Int J Clin Pharmacol Ther* 34:446
134. Maurer M, Henn V, Dittrich A, Hofmann A (1990) *Eur Arch Psychiatry Clin Neurosci* 240:1
135. Muller-Vahl KR, Schneider U, Kolbe H, Emrich HM (1999) *Am J Psychiatry* 156:495
136. Finnegan-Ling D, Musty RE (1994) Symposium on the cannabinoids, Burlington, Vermont. International Cannabinoid Research Society, p 53
137. Petro DJ, Ellenberger C Jr (1981) *J Clin Pharmacol* 21:413S
138. Merritt JC, McKinnon S, Armstrong JR, Hatem G, Reid LA (1980) *Ann Ophthalmol* 12:947
139. Jacob A, Todd AR (1940) *J Chem Soc*, p 649
140. Karniol IG, Carlini EA (1973) *Psychopharmacologia* 33:53
141. Jones G, Pertwee RG (1972) *Br J Pharmacol* 45:375
142. Zuardi AW, Morais SL, Guimaraes FS, Mechoulam R (1995) *J Clin Psychiatry* 56:485
143. Weiss L, Zeira M, Reich S, Har-Noy M, Mechoulam R, Slavín S, Gallily R (2006) *Autoimmunity* 39:143
144. Einhorn LH, Nagy C, Furnas B, Williams SD (1981) *J Clin Pharmacol* 21:64S

145. Aguiar AJ, Rasadi B (1983) US Patent 4406888. Pfizer, New York
146. Johnson MR, Melvin LS Jr (1983) US Patent 4371720. Pfizer, New York
147. Haubrich DR, Ward SJ, Baizman E, Bell MR, Bradford J, Ferrari R, Miller M, Perrone M, Pierson AK, Saelens JK, et al. (1990) *J Pharmacol Exp Ther* 255:511
148. D'Ambra TE, Estep KG, Bell MR, Eissenstat MA, Josef KA, Ward SJ, Haycock DA, Baizman ER, Casiano FM, Beblin NC, et al. (1992) *J Med Chem* 35:124
149. Rinaldi-Carmona M, Barth F, Millan J, Derocq JM, Casellas P, Congy C, Oustric D, Sarran M, Bouaboula M, Calandra B, Portier M, Shire D, Breliere JC, Le Fur GL (1998) *J Pharmacol Exp Ther* 284:644
150. Munjal M, ElSohly MA, Repka MA (2006) *AAPS Pharm Sci Tech* 7:71
151. van Drooge DJ, Hinrichs WL, Wegman KA, Visser MR, Eissens AC, Frijlink HW (2004) *Eur J Pharm Sci* 21:511
152. Perlin E, Smith CG, Nichols AI, Almirez R, Flora KP, Cradock JC, Peck CC (1985) *J Pharm Sci* 74:171
153. Hazekamp A, Ruhaak R, Zuurman L, Van Gerven J, Verpoorte R (2006) *J Pharm Sci* 95:1308
154. Valiveti S, Agu RU, Hammell DC, Paudel KS, Earles DC, Wermeling DP, Stinchcomb AL (2007) *Eur J Pharm Biopharm* 65:247
155. Mannila J, Jarvinen T, Jarvinen K, Tarvainen M, Jarho P (2005) *Eur J Pharm Sci* 26:71