Polyketide synthases in Cannabis sativa L

Flores-Sanchez, I.J.

Chapter I

Introduction to secondary metabolism in cannabis

Isvett J. Flores Sanchez • Robert Verpoorte

Pharmacognosy Department, Institute of Biology, Gorlaeus Laboratories, Leiden University
Leiden, The Netherlands
Published in Phytochem Rev (2008) 7:615–639

Cannabis sativa L. is an annual dioecious plant from Central Asia. Cannabinoids, flavonoids, stilbenoids, terpenoids, alkaloids and lignans are some of the secondary metabolites present in *C. sativa*. Earlier reviews focused on isolation and identification of more than 480 chemical compounds; this review deals with the biosynthesis of the secondary metabolites present in this plant. Cannabinoid biosynthesis and some closely related pathways that involve the same precursors are discussed.

I.1 Cannabis plant

Cannabis is an annual plant, which belongs to the family Cannabaceae. There are only 2 genera in this family: *Cannabis* and *Humulus*. While in *Humulus* only one species is recognized, namely *lupulus*, in *Cannabis* different opinions support the concepts for a mono or poly species genus.

Linnaeus (1753) considered only one species, *sativa*, however, McPartland *et al*. (2002) described 4 species, *sativa*, *indica*, *ruderalis* and *afghanica*; and Hillig (2005) proposed 7 putative taxa, *ruderalis*, *sativa* ssp. *sativa*, *sativa* ssp. *spontanea*, *indica* ssp. *kafiristanica*, *indica* ssp. *indica*, *indica* ssp. *afghanica* and *indica* ssp. *chinensis*. Nevertheless, the tendency in literature is to refer to all types of cannabis as *Cannabis sativa* L. with a variety name indicating the characteristics of the plant.

The cultivation of this plant, native from Central Asia, and its use has been spread all over the world by man since thousands of years as a source of food, energy, fiber and medicinal or narcotic preparations (Jiang *et al*., 2006; Russo, 2004; Wills, 1998).

Cannabis is a dioecious plant, i.e. it bears male and female flowers on separate plants. The male plant bears staminate flowers and the female plant pistillate flowers which eventually develop into the fruit and achenes (seeds). The sole function of male plants is to pollinate the females. Generally, the male plants commence flowering slightly before the females. During a few weeks the males produce abundant anthers that split open, enabling passing air currents to transfer the released pollen to the pistillate flowers. Soon after pollination, male plants wither and die, leaving the females maximum space, nutrients and water to produce a healthy crop of viable seeds. As result of special breeding, monoecious plants bearing both male and female flowers arose frequently in varieties developed for fiber production. The pistillate flowers consist of an ovary surrounded by a calyx with 2 pistils which trap passing pollen (Clarke, 1981; Raman, 1998). Each calyx is covered with glandular hairs (glandular trichomes), a highly specialized secretory tissue (Werker, 2000). In cannabis, these glandular trichomes are also present on bracts, leaves and on the underside of the anther lobes from male flowers (Mahlberg *et al*., 1984).

I.2 Secondary metabolites of Cannabis

The phytochemistry in cannabis is very complex; more than 480 compounds have been identified (ElSohly and Slade, 2005) representing different chemical classes. Some belong to primary metabolism, e.g. amino acids, fatty acids and steroids, while cannabinoids, flavonoids, stilbenoids, terpenoids, lignans and alkaloids represent secondary metabolites. The concentrations of these compounds depend on tissue type, age, variety, growth conditions (nutrition, humidity and light levels), harvest time and storage conditions (Keller *et al.*, 2001; Kushima *et al.*, 1980; Roos *et al.*, 1996). The production of cannabinoids increases in plants under stress (Pate, 1999). Ecological interactions have also been reported (McPartland *et al.*, 2000). Feeding studies in grasshoppers indicated that minimum amounts of cannabinoids are stored in their exoskeletons, being excreted in their frass (Rothschild *et al.*, 1977); although a neurotoxic activity was reported in midge larvaes using cannabis leaf extracts (Roy and Dutta, 2003).

I.2.1 Cannabinoids

This group represents the most studied compounds from cannabis. The term cannabinoid is given to the terpenophenolic compounds with 22 carbons (or 21 carbons for neutral form) of which 70 cannabinoids have been found so far and which can be divided into 10 main structural types (Figure 1). All other compounds that do not fit into the main types are grouped as miscellaneous (Figure 2). The neutral compounds are formed by decarboxylation of the unstable corresponding acids. Although decarboxylation occurs in the living plant, it increases during storage after harvesting, especially at elevated temperatures (Mechoulam and Ben-Shabat, 1999). Both forms are also further degraded into secondary products by the effects of temperature, light (Lewis and Turner, 1978) and auto-oxidation (Razdan *et al.*, 1972).

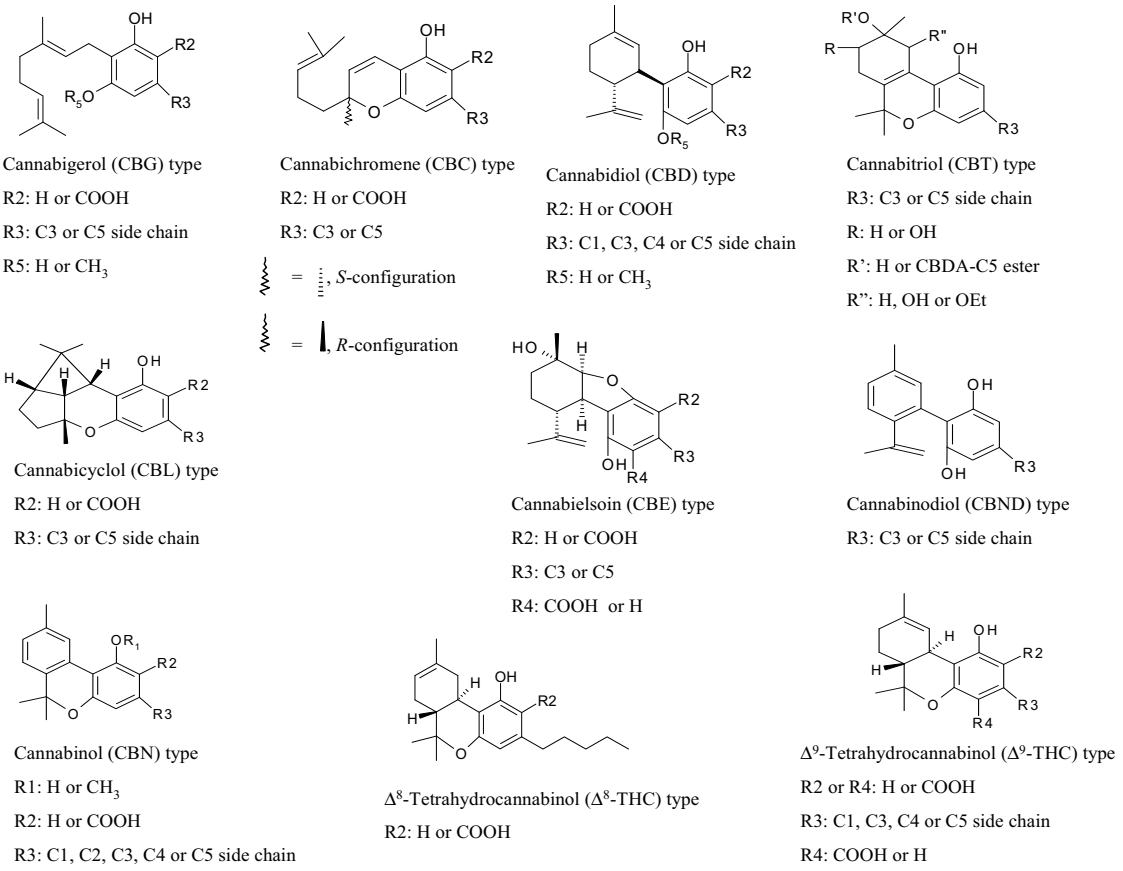


Figure 1. Cannabinoid structural types.

In cannabis, the most prevalent compounds are Δ^9-THC acid, CBD acid and CBN acid, followed by CBG acid, CBC acid and CBND acid, while the others are minor compounds. Based on the absolute concentration of Δ^9-THC (Δ^9-THC+ Δ^9-THC acid) and CBD (CBD + CBD acid) obtained via HPLC or GC analyses, the plants are classified as follows: Drug type (chemotype I), the concentration of Δ^9-THC is more than 2% and CBD concentration is less 0.5%; Fiber type (chemotype III), the Δ^9-THC concentration is less than 0.3% and the concentration of CBD is more than 0.5%; Intermediate type (chemotype II), the concentrations of both are similar, usually more than 0.5% for each; and Propyl isomer/C3 type (chemotype IV), which can be differentiated by the dominant key cannabinoids Δ^9-tetrahydrocannabivarinic acid (Δ^9-THCVA) and Δ^9-tetrahydrocannabivarin (Δ^9-THCV), while also containing considerable amounts of Δ^9-THC (Brenneisen and ElSohly, 1988; Fournier *et al.*, 1987; Lehmann and Brenneisen, 1995).

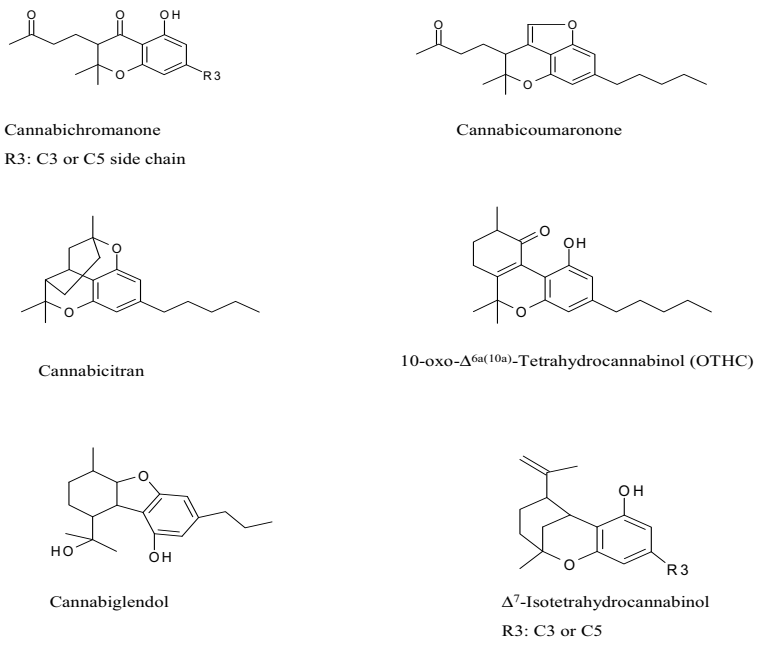


Figure 2. Miscellaneous cannabinoids.

The psychotropic activities of cannabinoids are well known (Paton and Pertwee, 1973; Ranganathan and D'Souza, 2006); however, in clinical studies, *in vitro* and *in vivo*, some other pharmacological effects of cannabinoids are observed such as antinociceptive, antiepileptic, cardiovascular, immunosuppressive (Ameri, 1999), antiemetic, appetite stimulation (Mechoulam and Ben Shabat, 1999), antineoplastic (Carchman *et al.*, 1976; Massi *et al.*, 2004), antimicrobial (ElSohly *et al.*, 1982), anti-inflammatory (Formukong *et al.*, 1988), neuroprotective antioxidants (Hampson *et al.*, 1988) and positive effects in psychiatric syndromes, such as depression, anxiety and sleep disorders (Grotenhermen, 2002; Musty, 2004). These effects could be due to agonistic nature of these compounds with respect to the cannabinoid CB_1- and CB_2 receptors (Matsuda *et al.*, 1990; Munro *et al.*, 1993) which compete with endocannabinoids (Mechoulam *et al.*, 1998), a family of cannabinoid receptor ligands participating in modulation of neurohumoral activity (Di Marzo *et al.*, 2007; Giuffrida *et al.*, 1999; Velasco *et al.*, 2005). Some therapeutic applications from cannabis, cannabinoids, cannabinoid analogs and CB receptor agonist/antagonist are shown in table 1.

Table 1. Some pharmacological applications of medicinal cannabis, THC, analogs and others.

Product	Components/ active ingredient	Prescription/ clinical effects	Administering	Country	Reference/ Company
Cannabis flos variety Bedrocan®	Dry flowers, 18% Δ^9-THC and 0.2% CBD	Spasticity with pain in MS or spinal cord injury; nausea and vomiting by radiotherapy, chemotherapy and HIV-medication; chronic neuralgic pain and Gilles de la Tourette Syndrome; palliative treatment of cancer and HIV/AIDS	Smoking	NL	Office of Medicinal Cannabis (OMC)
Cannabis flos variety Bedrobinol®	Dry flowers, 13% Δ^9-THC and 0.2% CBD	Spasticity with pain in MS or spinal cord injury; nausea and vomiting by radiotherapy, chemotherapy and HIV-medication; chronic neuralgic pain and Gilles de la Tourette Syndrome; palliative treatment of cancer and HIV/AIDS	Smoking	NL	Office of Medicinal Cannabis (OMC)
Marinol®	synthetic THC (capsules)	Nausea and vomiting by chemotherapy; appetite loss associated with weight loss by HIV/AIDS	Oral	USA	Solvay Pharmaceuticals, Inc.
Sativex®	Cannabis extract, 27 mg/ml Δ^9-THC and 25 mg/ml CBD	Neuropathic pain in MS	Oromucosal	Canada	GW Pharm Ltd.
Cesamet™	THC analog (capsules)	Nausea and vomiting by cancer chemotherapy	Oral	USA	Valeant Pharmaceuticals International
Ajulemic acid (CT-3)	Δ^8-THC-11-oic acid** analog, CB_1 and CB_2 agonist	Analgesic effect in chronic neuropathic pain	Oral	-	Karst *et al*., 2003
Dexanabinol (HU-211)	11-OH-Δ^8-THC* analog, *N*-methyl-D-aspartate antagonist	Neuroprotection	Intravenous	-	Knoller *et al*., 2002/ Pharmos Ltd.
Rimonabant/ Acomplia® (SR141716A)	NPCDMPCH, CB_1 selective antagonist	Adjunct to diet and exercise in the treatment of obese or overweight patients with associated risk factors such as type II diabetes or dyslipidaemia	Oral	Europe	Van Gall *et al*., 2005; Aronne, 2007; Henness *et al*., 2006 / Sanofi-Aventis

MS, Multiple Sclerosis; AIDS, acquired immunodeficiency syndrome; NL, The Netherlands
NPCDMPCH, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2, 4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride
* 11-OH-Δ^8-THC is primary metabolite from Δ^8-THC, which is further metabolized to ** Δ^8-THC-11-oic acid by hepatic cytochrome P450s in humans

Table 2. Identified enzymes from cannabinoid pathway.

Enzyme	Source	MW (kDa)	*Km* (µM) substrate	pH opt.	pI	V_{max} (nkat/ mg)	K_{cat} (s^{-1})		(Sp activity, pKat/mg)		nce
Olivetol synthase	Flower, Leaf		- Mal-CoA Hex-CoA	6.8					partially	olivetol	Raharjo *et al.* 2004a
Geranyl diphosphate :olivetolate geranyltransferase (GOT)	Leaf		2000 GPP Olivetolic acid	7.0				Mg^{+2}, ATP	partially	CBGA	Fellermeier and Zenk 1998
			- NPP Olivetolic acid	7.0				Mg^{+2}, ATP	partially	*trans*-CBGA	Fellermeier and Zenk 1998
CBCA synthase	Leaf	71	23 CBGA	6.5	7.3	0.67	0.04		homogeneity (607)	CBCA	Morimoto *et al.* 1998
CBDA synthase	Leaf	74	137 CBGA	5.0	6.1	2.57	0.19		homogeneity (1510)	CBDA	Taura *et al.* 1996
			206 *trans*-CBGA	5.0		0.39	0.03		homogeneity	CBDA	Taura *et al.* 1996
Δ^9-THCA synthase	Leaf	75	134 CBGA	6.0	6.4	2.68	0.2		homogeneity	Δ^9-THCA	Taura *et al.* 1995a
Δ^9-THCA synthase	Leaf (recombinant tobacco hairy roots)	58.6	- CBGA	5.0					homogeneity	Δ^9-THCA	Sirikantaramas *et al.* 2004
	Leaf (recombinant insect cells)	60	540 CBGA	5.0			0.3	FAD, O_2	homogeneity	Δ^9-THCA	Sirikantaramas *et al.* 2004

CBCA, cannabichromenic acid; CBDA, cannabidiolic acid; CBGA, cannabigerolic acid; Δ^9-THCA, Δ^9-tetrahydrocannabinolic acid; Mal-CoA, malonyl-CoA; Hex-CoA, hexanoyl-CoA; GPP, geranyl diphosphate

I.2.1.1 Cannabinoid biosynthesis

Histochemical (André and Vercruysse, 1976; Petri *et al.*, 1988), immunochemical (Kim and Mahlberg, 1997) and chemical (Lanyon *et al.*, 1981) studies have confirmed that glandular hairs are the main site of cannabinoid production, although they have also been detected in stem, pollen, seeds and roots by immunoassays (Tanaka and Shoyama, 1999) and chemical analysis (Potter, 2004; Ross *et al.*, 2000).

The precursors of cannabinoids are synthesized from 2 pathways, the polyketide pathway (Shoyama *et al.*, 1975) and the deoxyxylulose phosphate/methyl-erythritol phosphate (DOXP/MEP) pathway (Fellermeier *et al.*, 2001) (Figure 3). From the polyketide pathway, olivetolic acid is derived and from the DOXP/MEP pathway, geranyl diphosphate (GPP) is derived. Both are condensed by the prenylase geranyl diphosphate:olivetolate geranyltransferase (GOT) (Fellermeier and Zenk, 1998) to form cannabigerolic acid (CBGA), which is a common substrate for three oxydocyclases: Cannabidiolic acid synthase (Taura *et al.*, 1996), Δ^9-Tetrahydrocannabinolic acid synthase (Taura *et al.*, 1995a) and Cannabichromenic acid synthase (Morimoto *et al.*, 1998), forming cannabidiolic acid (CBDA), Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA) and cannabichromenic acid (CBCA), respectively (Morimoto *et al.*, 1999).

It is known that prenyltransferases condense an acceptor isoprenoid or non-isoprenoid molecule to an allylic diphosphate and depending on their specificities these prenyltransferases yield linear *trans*- or *cis*- prenyl diphosphates (Bouvier *et al.*, 2005). From *in vitro* assays it was observed that GOT could accept neryl diphosphate (NPP), the isomer of GPP which is formed by an isomerase (Shine and Loomis, 1974), as a substrate forming cannabinerolic acid (*trans*-CBGA) (Fellermeier and Zenk, 1998); this isomer of CBGA could be transformed to CBDA by a CBDA synthase (Taura *et al.*, 1996). The presence of *trans*-CBGA in cannabis has been shown (Taura *et al.*, 1995b). Probably, more than one enzymatic isoform coexist. It is known that depending on its degree of connectivity within the metabolic network, multiple isoforms of the same enzyme could preserve the integrity of the metabolic network; e.g. in the face of mutation. It has also been suggested that different organizations or associations from isoforms of the key biosynthetic enzymes into a metabolon, a

complex of sequential metabolic enzymes, could be differentially regulated (Jorgensen *et al.*, 2005; Sweetlove and Fernie, 2005).

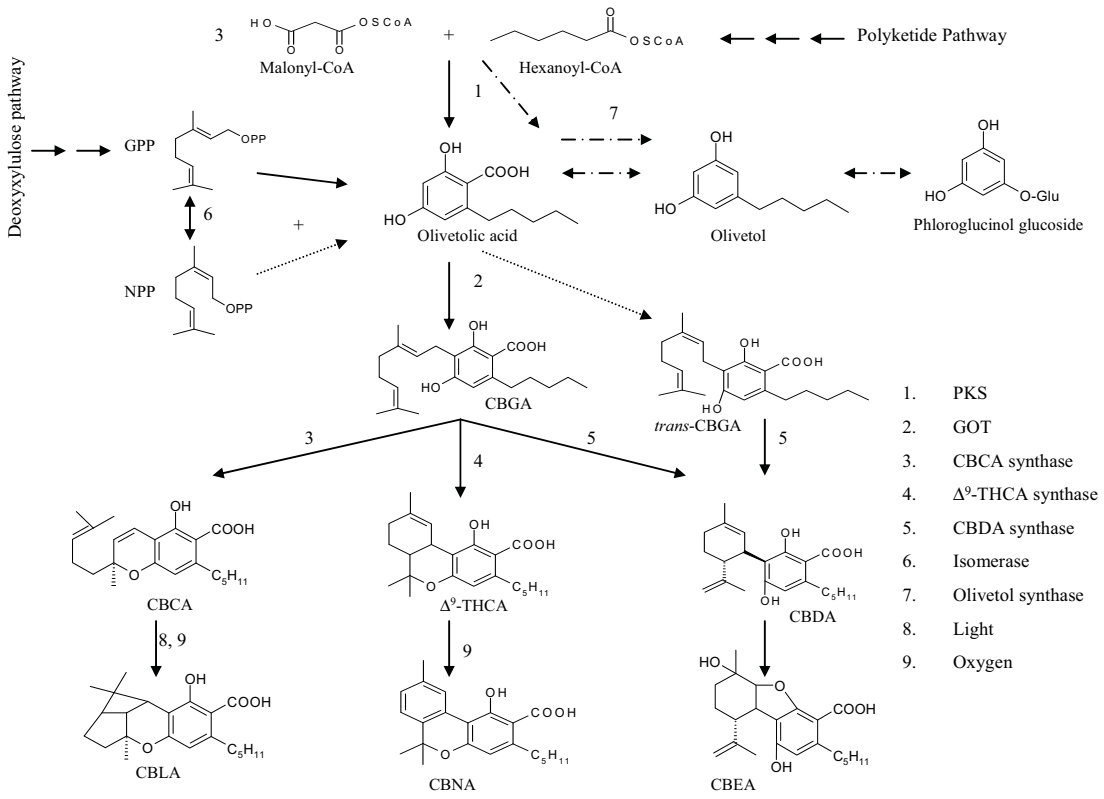


Figure 3. General overview of biosynthesis of cannabinoids and putative routes.

In table 2, some characteristics of the studied enzymes from the cannabinoid route are shown. The gene that encodes the enzyme THCA synthase has been cloned (Sirikantaramas *et al*., 2004) and consists of a 1635-bp open reading frame, which encodes a polypeptide of 545 amino acids. The expressed protein revealed that the reaction is FAD-dependent and the binding of a FAD molecule to the histidine-114 residue is crucial for its activity. From the deduced amino acid sequence a cleavable signal peptide and glycosylation sites were found; suggesting post-translational regulation of the protein (Huber and Hardin, 2004; Uy and Wold, 1977). In addition, it was shown that THCA synthase is expressed exclusively in the glandular hairs and is also a secreted biosynthetic enzyme, which was localized to and functioned in the storage cavity of the glandular hairs; indicating that the storage cavity is not only the site for the accumulation of cannabinoids but also for the biosynthesis of THCA (Sirikantaramas *et al*., 2005). This enzyme also has been crystallized (Shoyama *et al*., 2005). The CBDA synthase gene has been cloned and expressed (Taura *et al*., 2007b); the open reading frame encodes a 544 amino acid polypeptide, showing 83.9% of homology with THCA synthase. Furthermore, the expressed protein revealed a FDA-dependent reaction similar to THCA synthase and glycosylation sites were also found. In addition, it was suggested that a difference between the two reaction mechanisms from THCA and CBDA synthases is seen in the proton transfer step; while CBDA synthase removes a proton from the terminal methyl group of CBGA, THCA synthase takes it from the hydroxyl group of CBGA.

The transformation from CBD to CBE by cannabis suspension (Hartsel *et al*., 1983), callus cultures (Braemer *et al*, 1985) and *Saccharum officinarum* L. cultures (Hartsel *et al*., 1983) have been reported, as well as the transformation of Δ^9-THC to cannabicoumaronone (Braemer and Paris, 1987) by cannabis cell suspension cultures. From these studies, an epoxidation by epoxidases or cytochromes P-450 enzymes was proposed or a free radical-mediated oxidation mechanism (reactive oxygen species, ROS). It should be noted that the mentioned bioconversions all concern the decarboxylated compounds, i.e. not the normal biosynthetic products in the plant. Studies on the corresponding acids are required to reveal any relationship between the bioconversion experiments and the cannabinoid biosynthesis.

Oxidative stress in plants can be induced by several factors such as anoxia or hypoxia (by excess of rainfall, winter ice encasement, spring floods, seed imbibition, etc.), pathogen invasion, UV stress, herbicide action and programmed cell death or senescence (Blokhina *et al*., 2003; Jabs, 1999; Pastori and del Rio, 1997). The proposed mechanisms of oxidation from the neutral and acid forms of Δ^9-THC to the neutral and acid forms of CBN or Δ^8-THC by free radicals or hydroxylated intermediates (Miller, *et al*., 1982; Turner and ElSohly, 1979) could originate from a production of ROS. Antioxidants and antioxidant enzymes such as tocopherols, phenolic compounds (flavonoids), superoxide dismutase, ascorbate peroxidase and catalase have been proposed as components of an antioxidant defense mechanism to control the level of ROS and protect cells under stress conditions (Blokhina *et al*., 2003). Cannabinoids could fit in this antioxidant system, however, their specific accumulation in specialized glandular cells point to another function for these compounds, e.g. antimicrobial agent. Sirikantaramas *et al*. (2005) found that cannabinoids are cytotoxic compounds for cell suspension cultures from *C. sativa*, tobacco BY-2 and insects; suggesting that the cannabinoids act as plant defense compounds and would protect the plant from predators such as insects. The THCA synthase reaction produces hydrogen peroxide as well as THCA during the oxidation of CBGA (Sirikantaramas *et al*., 2004), a toxic amount of hydrogen peroxide could be accumulated together with the cannabinoids which must be secreted into the storage cavity from the glandular hairs to avoid cellular damage itself. Additionally, Morimoto *et al*. (2007) have shown that cannabinoids have the ability to induce cell death through mitochondrial permeability transition in cannabis leaf cells, suggesting a regulatory role in cell death as well as in the defense systems of cannabis leaves. On the other hand, although CBN type cannabinoids have been isolated from cannabis extracts, they are probably artifacts (ElSohly and Slade, 2005).

Feeding studies using cannabigerovarinic acid (CBGVA) as precursor, showed that the biosynthesis of propyl cannabinoids (Shoyama *et al*., 1984) probably follows a similar pathway (Figure 4) yielding cannabidivarinic acid (CBDVA), cannabichromevarinic acid (CBCVA), Δ^9-tetrahydrocannabivarinic acid (Δ^9-THCVA), cannabielsovarinic acid B (CBEVA-B) and cannabivarin (CBV).

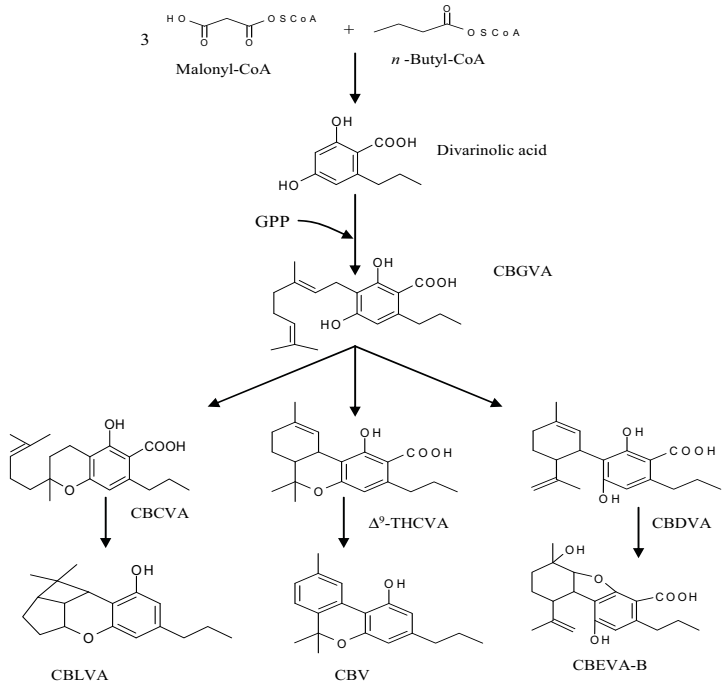


Figure 4. Proposed biogenetic pathway for cannabinoids with C3 side-chain.

Based on the structure of olivetolic acid (Figure 3), a polyketide synthase (PKS) could be involved in its biosynthesis. Raharjo *et al*. (2004a) found *in vitro* enzymatic activity for a PKS, though yielding the olivetol and not the olivetolic acid as the reaction product. It is known that olivetolic acid is the active form for the next biosynthetic reaction steps of the cannabinoids. Feeding studies (Kajima and Piraux, 1982), however, showed a low incorporation in cannabinoids using radioactive olivetol as precursor. Studies on the isoprenoid pathway suggest that the flux of active precursors (prenyl diphosphates) can be stopped by enzymatic hydrolysis by phosphatases, activated by kinases or even redirected to other biosynthetic processes (Goldstein and Brown, 1990; Meigs and Simoni, 1997). Furthermore, the presence of phloroglucinol glucoside in cannabis (Hammond and Mahlberg, 1994) suggests a regulatory role for olivetolic acid in the biosynthesis of cannabinoids (Figure 3), although, the presence of olivetolic acid and olivetol in ants from genus *Crematogaster* has been reported (Jones *et al*., 2005); both olivetolic acid and olivetol are classified as resorcinolic lipids (alkylresorcinol, resorcinolic acid); these last ones have

been detected in several plants and microorganisms (Roos *et al.*, 2003; Jin and Zjawiony, 2006).

Kozubek and Tyman (1999) suggested that alkylresorcinols, such as olivetol, are formed from biosynthesized alkylresorcinolic acids by enzymatic decarboxylation or via modified fatty acid-synthesizing enzymes, where the alkylresorcinolic acid carboxylic group would be expected to be also attached either to ACP (acyl carrier protein) or to CoA. Thus, in the release of the molecule from the protein compartment in which it was attached or elongated, simultaneous decarboxylation of the alkylresorcinol may occur, otherwise the alkylresorcinolic acid would be the final product. Recently, it was shown that the fatty acid unit acts as a direct precursor and forms the side-chain moiety of alkylresorcinols (Suzuki *et al.*, 2003). The identification of methyl- (Vree *et al.*, 1972), butyl- (Smith, 1997), propyl- and pentyl-cannabinoids suggests the biosynthesis of alkylresorcinolic acids with different side-chain moieties, originating from different lengths of an activated short chain fatty acid unit (fatty acid-CoA). This side chain is important for the affinity, selectivity and pharmacological potency for the cannabinoids receptors (Thakur *et al.*, 2005).

Biotransformation of cannabinoids to glucosylated forms by plant tissues (Tanaka *et al.*, 1993; Tanaka *et al.*, 1996; Tanaka *et al.*, 1997) and various oxidized derivatives by microorganisms (Binder and Popp, 1980; Robertson *et al.*, 1978) have been reported; as well as biotransformations for olivetol (McClanahan and Robertson, 1984). However, the best studied biotransformations are in animals and humans (Mechoulam, 1970; Watanabe *et al.*, 2007)

I.2.2 Flavonoids

Flavonoids are ubiquitous and have many functions in the biochemistry, physiology and ecology of plants (Shirley, 1996; Gould and Lister, 2006), and they are important in both human and animal nutrition and health (Manthey and Buslig, 1998; Ferguson, 2001). In cannabis, more than 20 flavonoids have been reported (Clark and Bohm, 1979, Vanhoenacker *et al.*, 2002; ElSohly and Slade, 2005) representing 7 chemical structures which can be glycosylated, prenylated or methylated (Figure 5) Cannflavin A and cannflavin B are methylated isoprenoid flavones (Barron and Ibrahim, 1996). Some pharmacological effects from cannabis flavonoids have been detected such as inhibition of

prostaglandin E_2 production by cannaflavin A and B (Barrett *et al.*, 1986), inhibition of the activity of rat lens aldose reductase by *C*-diglycosylflavones, orientin and quercetin (Segelman *et al.*, 1976); other studies only suggest a possible modulation with the cannabinoids (McPartland and Mediavilla, 2002).

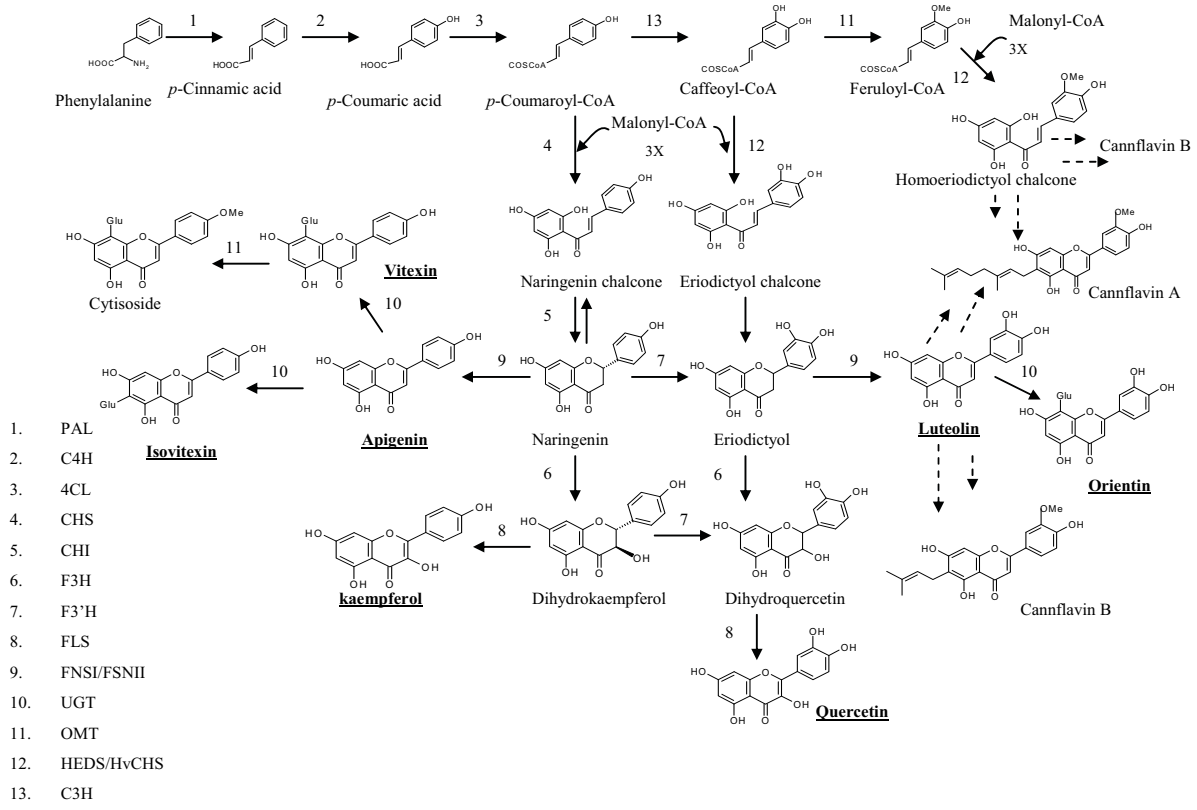


Figure 5. Proposed general phenylpropanoid and flavonoid biosynthetic pathways in *Cannabis sativa.* C3H, *p*-coumaroyl-CoA 3-hydroxylase; main structures of flavones and flavonols are in bold and underlined.

I.2.2.1 Flavonoid biosynthesis

Cannabis flavonoids have been isolated and detected from flowers, leaves, twigs and pollen (Segelman *et al.*, 1978; Vanhoenacker *et al.*, 2002; Ross *et al.*, 2005). There is no evidence indicating the presence of flavonoids in glandular trichomes, however, it is know that in *Betulaceae* family and in the genera *Populus* and *Aesculus* flavonoids are secreted by glandular trichomes or by a secretory epithelium (Wollenweber, 1980). Acylated kaempferol glycosides have

also been detected in leaf glandular trichomes from *Quercus ilex* (Skaltsa *et al.*, 1994), and flavone aglycones from *Origanum* x *intercedens* (Bosabalidis *et al.*, 1998) and from *Mentha* x *piperita* (Voirin *et al.*, 1993).

Although the flavonoid pathway has been extensively studied in several plants (Davies and Schwinn, 2006), there is no data on the biosynthesis of flavonoids in cannabis. The general pathway for flavone and flavonol biosynthesis as it is expected to occur in cannabis is shown in figure 5. The precursors are phenylalanine from the shikimate pathway and malonyl-CoA, which is synthesized by carboxylation of acetyl-CoA, a central intermediate in the Krebs tricarboxylic acid cycle (TCA cycle). Phenylalanine is converted into *p*-cinnamic acid by a Phenylalanine ammonia lyase (PAL), EC 4.3.1.5; this *p*-cinnamic acid is hydroxylated by a Cinnamate 4-hydroxylase (C4H), EC 1.14.13.11, to *p*-coumaric acid and a CoA thiol ester is added by a 4-Coumarate:CoA ligase (4CL), EC 6.2.1.12. One molecule of *p*-coumaroyl-CoA and three molecules of malonyl-CoA are condensed by a Chalcone synthase (CHS), EC 2.3.1.74, a PKS, yielding naringenin chalcone. The naringenin chalcone is subsequently isomerized by the enzyme Chalcone isomerase (CHI), EC 5.5.1.6, to naringenin, a flavanone. This naringenin is the common substrate for the biosynthesis of flavones and flavonols. Hydroxy substitution to ring C at position 3 by a Flavanone 3-hydrolase (F3H), EC 1.14.11.9; and to ring B at position 3' by a Flavonoid 3'-hydrolase (F3'H), EC 1.14.13.21, occurs in naringenin. F3H is a 2-oxoglutarate-dependent dioxygenase (2OGD) and F3'H is a cytocrome P450. Subsequently, in the ring C at positions 2 and 3 a double bond is formed by a Flavonol synthase (FLS), EC 1.14.11.-, or Flavone synthase (FNS). FLS is a 2ODG and for FNS two distinct activities have been characterized that convert flavanones to flavones. In most plants FNS is a P450 enzyme (FNSII, EC 1.14.13.-), but in species from Apiaceae family FNS is a 2ODG (FNSI, EC 1.14.11.-). Modification reactions as glycosylation by UDP-glycosyltransferase (UGT, EC 2.4.1,-), methylation by a SAM-methyltransferase (OMT, EC 2.1.1.-) and prenylation by prenyltransferases are added to the flavone and flavonol. Alternative routes for luteolin, and cannflavin A / B biosynthesis starting from feruloyl-CoA or caffeoyl-CoA with malonyl-CoA are also proposed. Conversion of these substrates to homoeriodictyol or eriodictyol by Homoeriodictyol/eriodictyol synthase (HEDS or HvCHS), a PKS, has been shown (Christensen *et al.*, 1998). Feruloyl-CoA and caffeoyl-CoA are phenylpropanoids

which are derivatives from *p*–coumaric acid and are precursors for lignin biosynthesis (Douglas, 1996). HvCHS leads the production of the methylated flavanone homoeriodictyol and eliminate the need of the F3'H and the OMT. It has been shown that the flavonoid pathway is tightly regulated and several transcription factors have been identified (Davies and Schwinn, 2003; Davies and Schwinn, 2006), as well as formation of metabolons (Winkel–Shirley, 1999).

From biotransformation studies using *C. sativa* cell cultures, the transformation from apigenin to vitexin was shown, as well as glycosylations from apigenin to apigenin 7–*O*–glucoside and from quercetin to quercetin–*O*–glucoside (Braemer *et al.*, 1986). Regarding to PKS in cannabis, CHS activity was detected from flower protein extracts (Raharjo *et al.*, 2004a) and one PKS gene from leaf was identified (Raharjo *et al.*, 2004b), which expressed activity for CHS, Phlorisovalerophenone synthase (VPS) and Isobutyrophenone synthase (BUS). VPS, isolated from *H. lupulus* L. cones (Paniego *et al.*, 1999), and BUS, isolated from *Hypericum calycinum* cell cultures (Klingauf *et al.*, 2005), are PKSs that condense malonyl–CoA with isovaleryl–CoA or isobutyryl–CoA, respectively.

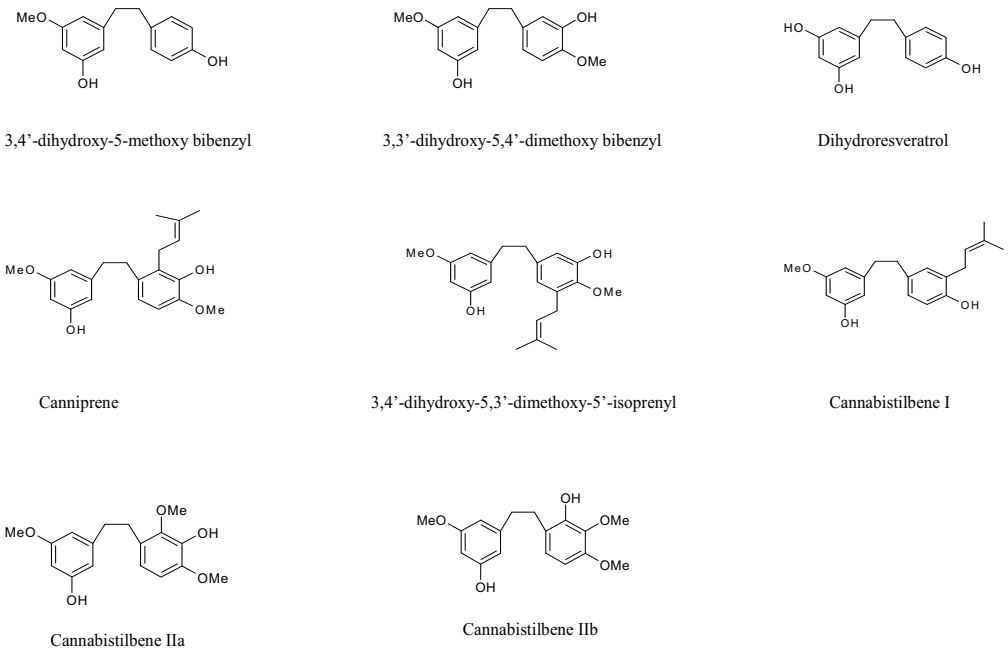
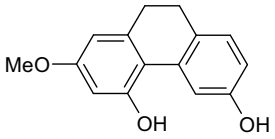


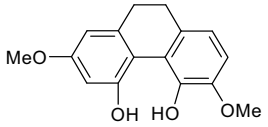
Figure 6. Bibenzyls compounds in *C. sativa*. The configuration of the structures is not given for simplicity reasons.

I.2.3 Stilbenoids

The stilbenoids are phenolic compounds distributed throughout the plant kingdom (Gorham *et al.*, 1995). Their functions in plants include constitutive and inducible defense mechanisms (Chiron *et al.*, 2001; Jeandet *et al.*, 2002), plant growth inhibitors and dormancy factors (Gorham, 1980). Frequently, the stilbenoids are constituents of heartwood or roots, and have antifungal and antibacterial activities (Kostecki *et al.*, 2004; Vastano *et al.*, 2000) or they are repellent towards insects (Hillis and Inoue, 1968). Nineteen stilbenoids have been identified in cannabis (Ross and ElSohly, 1995; Turner *et al.*, 1980) (Figures 6-8).



Cannithrene 1



Cannithrene 2

Figure 7. Spirans from *C. sativa*. A, 7-hydroxy-5-methoxyindan-1-spiro-cyclohexane; B, 5-hydroxy-7-methoxyindan-1-spiro cyclohexane; C, 5,7-dihydroxyindan-1-spiro-cyclohexane.

Although some studies have reported antibacterial activity for some cannabis stilbenoids (Molnar *et al.*, 1985) others have reported that the cannabis bibenzyls 3,4'-dihydroxy-5-methoxybibenzyl, 3,3'-dihydroxy-5,4' -dimethoxybibenzyl, 3,4'-dihydroxy-5,3'-dimethoxy-5'-isoprenyl bibenzyl did not shown activity in bactericidal, estrogenic and, germination- and growth-inhibiting properties or the SINDROOM tests (a screening test for central nervous system activity) (Kettenes-van den Bosch, 1978).

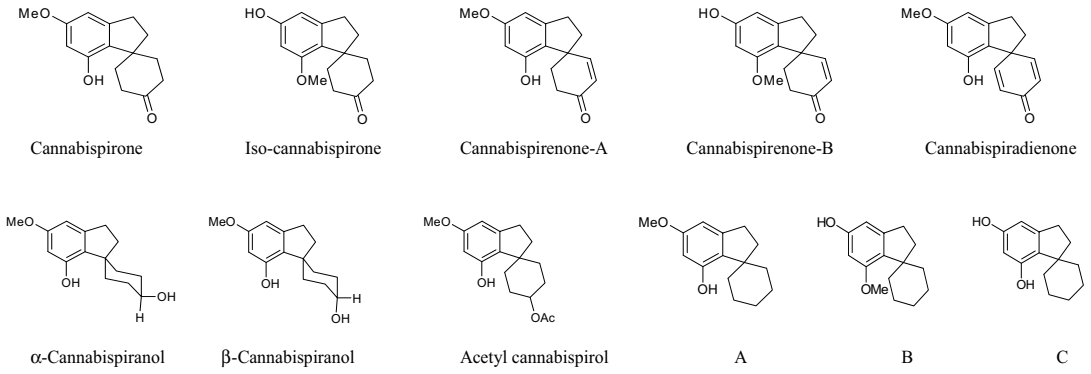


Figure 8. Spirans from *C. sativa*. A, 7-hydroxy-5-methoxyindan-1-spiro-cyclohexane; B, 5-hydroxy-7-methoxyindan-1-spiro cyclohexane; C, 5,7-dihydroxyindan-1-spiro-cyclohexane.

It has been observed that the stilbenoids show activities such as anti-inflammatory (Adams *et al.*, 2005; Djoko *et al.*, 2007; Leiro *et al.*, 2004), antineoplastic (Iliya *et al.*, 2006; Oliver *et al.*, 1994; Yamada *et al.*, 2006), neuroprotective (Lee *et al.*, 2006), cardiovascular protective (Leiro *et al.*, 2005; Estrada-Soto *et al.*, 2006), antioxidant (Stivala *et al.*, 2001) antimicrobial (Lee *et al.*, 2005), and longevity agents (Kaeberlein *et al.*, 2005; Valenzano *et al.*, 2006).

I.2.3.1 Stilbenoid biosynthesis

Cannabis stilbenoids have been detected and isolated from stem (Crombie and Crombie, 1982), leaves (Kettenes-van den Bosch and Salemink, 1978) and resin (El-Feraly *et al.*, 1986).

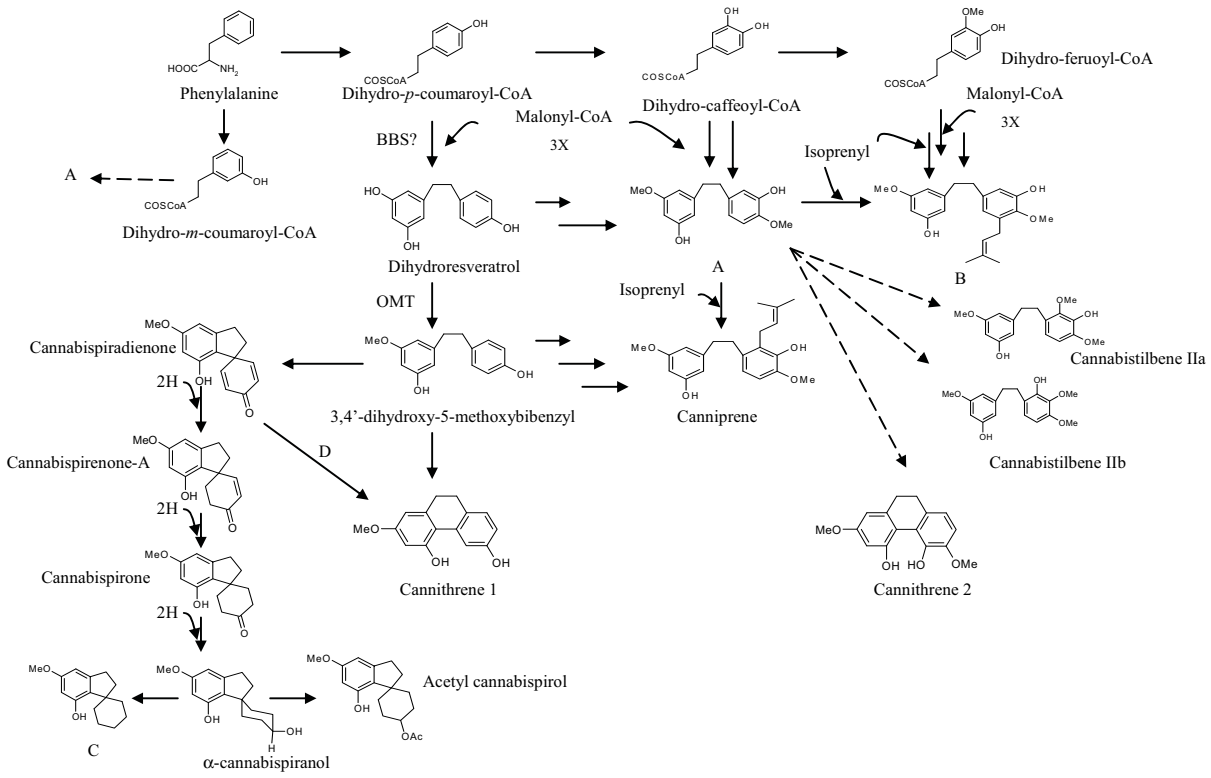


Figure 9. Proposed pathway for the biosynthesis of stilbenoids in *C. sativa*. A) 3,3'-dihydroxy-5,4'-dimethoxybibenzyl; B) 3,4'-dihydroxy-5,3'-dimethoxy-5'-isoprenylbibenzyl;C) 7-hydroxy-5-methoxyindan-1-spiro-cyclohexane; D) Dienone-phenol *in vitro* rearrangement (heat, acidic pH).

It has been suggested (Crombie and Crombie, 1982; Shoyama and Nishioka, 1978) that their biosynthesis could have a common origin (Figure 9). The first step could be the formation of bibenzyl compounds from the condensation of one molecule of dihydro-*p*-coumaroyl-CoA and 3 molecules of malonyl-CoA to dihydroresveratrol. It was shown that in cannabis both dihydroresveratrol and canniprene are synthesized from dihydro-*p*-coumaric acid (Kindl, 1985). In orchids, the induced synthesis by fungal infection of bibenzyl compounds by a PKS, called Bibenzyl synthase (BBS), was shown to condense dihydro-*m*-coumaroyl-CoA and malonyl-CoA to 3,3',5-trihydroxybibenzyl (Reinecke and Kindl, 1994a). It was also found that this enzyme can accept dihydro-*p*-coumaroyl-CoA and dihydrocinnamoyl-CoA as substrates, although to a lesser degree. Dihydropinosylvin synthase is an enzyme from *Pinus sylvestris* (Fliegmann *et al.*, 1992) that accepts dihydrocinnamoyl-CoA as substrate to form bibenzyl dihydropinosylvin. Gehlert and Kindl (1991) found a relationship

between induced formation by wounding of 3,3'-dihydroxy-5,4'-dimethoxybibenzyl and the enzyme BBS in orchids. This result also suggests that in cannabis the 3,3'-dihydroxy-5,4'-dimethoxybibenzyl compound could have the 3,3',5-trihydroxybibenzyl formed from dihydro-*m*-coumaroyl-CoA or dihydro-caffeoyl-CoA as intermediate. In orchids, however, the incorporation of phenylalanine into dihydro-*m*-coumaric acid, dihydrostilbene and dihydrophenanthrenes was shown (Fritzemeier and Kindl, 1983); indicating an origin from the phenylpropanoid pathway. Similar to flavonoid biosynthesis, modification reactions such as methylation and prenylation could form the rest of the bibenzyl compounds in cannabis. A second step could involve the synthesis of 9,10-dihydrophenanthrenes from bibenzyls. It is known that *O*-methylation is a prerequisite for the cyclization of bibenzyls to dihydrophenanthrenes in orchids (Reinecke and Kindl, 1994b) and a transient accumulation of the mRNAs from *S*-adenosyl-homocysteine hydrolase and BBS was also detected upon fungal infection (Preisig-Müller *et al.*, 1995). The cyclization mechanism in plants is unknown. An intermediate step between bibenzyls and 9,10-dihydrophenanthrenes could be involved in the biosynthesis of spirans. It has been proposed that spirans could be derived from *o*-*p*, *o*-*o* or *p*-*p* coupling of dihydrostilbenes followed by reduction (Crombie, 1986; Crombie *et al.*, 1982) and that 9,10-dihydrophenanthrenes could be derived by a dienone-phenol rearrangement from the spirans. No reports about the biosynthesis of spirans or about the regulation of the stilbenoid pathway in cannabis exist.

I.2.4 Terpenoids

The terpenoids or isoprenoids are another of the major plant metabolite groups. The isoprenoid pathway generates both primary and secondary metabolites (McGarvey and Croteau, 1995). In primary metabolism the isoprenoids have functions as phytohormones (gibberellic acid, abscisic acid and cytokinins) and membrane stabilizers (sterols), and they can be involved in respiration (ubiquinones) and photosynthesis (chlorophylls and plastoquinones); while in secondary metabolism they participate in the communication and plant defense mechanisms (phytoalexins). In cannabis 120 terpenes have been identified (ElSohly and Slade, 2005): 61 monoterpenes, 52 sesquiterpenoids, 2 triterpenes, one diterpene and 4 terpenoid derivatives

(Figure 10). The terpenes are responsible for the flavor of the different varieties of cannabis and determine the preference of the cannabis users. The sesquiterpene caryophyllene oxide is the primary volatile detected by narcotic dogs (Stahl and Kunde, 1973). It has been observed that terpene yield and floral aroma vary with the degree of maturity of female flowers (Mediavilla and Steinemann, 1997) and it has been suggested that terpene composition of the essential oil could be useful for the chemotaxonomic analysis of cannabis plants (Hillig, 2004). Pharmacological effects have been detected for some cannabis terpenes and they may synergize the effects of the cannabinoids (Burstein *et al.*, 1975; McPartland and Mediavilla, 2002). Terpenes have been detected and isolated from the essential oil from flowers (Ross and ElSohly, 1996), roots (Slatkin *et al.*, 1971) and leaves (Bercht *et al.*, 1976; Hendriks *et al.*, 1978); however, the glandular hairs are the main site of localization (Malingre *et al.*, 1975).

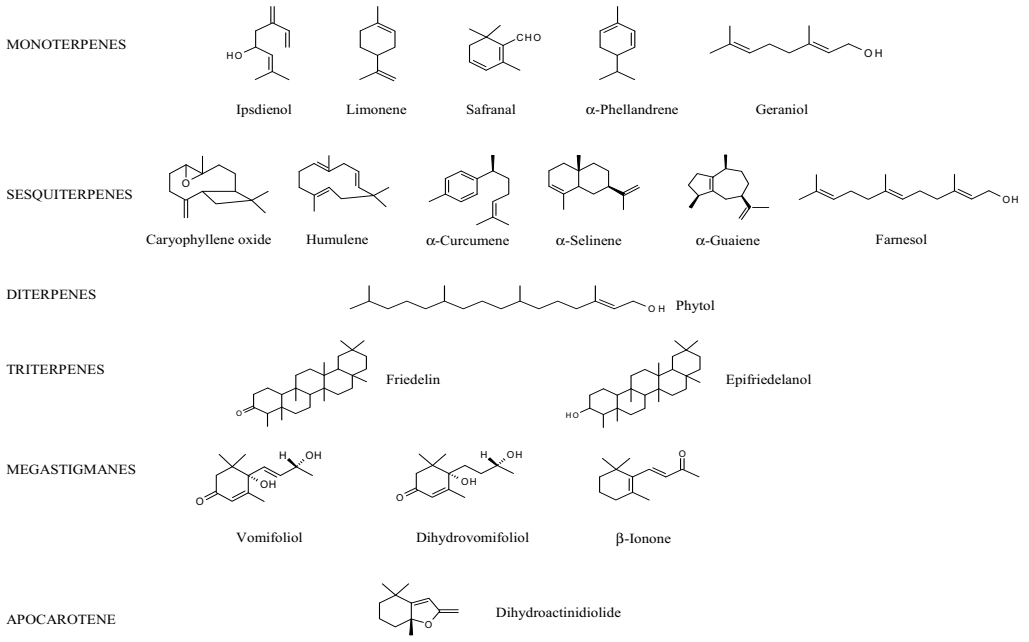


Figure 10. Some examples of isolated terpenoids from *C. sativa*.

I.2.4.1 Terpenoid biosynthesis

The isoprenoid pathway has been extensively studied in plants (Bouvier *et al.*, 2005). The terpenoids are derived from the mevalonate (MVA) pathway, which is active in the cytosol, or from the plastidial deoxyxylulose phosphate/methyl-

erythritol phosphate (DOXP/MEP) pathway (Figure 11). Both pathways form isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). Condensation reactions by prenyl transferases produce a series of prenyl diphosphates. Generally, it is considered that the MVA pathway provides precursors for the synthesis of sesquiterpenoids, triterpenoids, steroids and others; while the DOXP/MEP pathway supplies precursors for monoterpenoids, diterpenoids, carotenoids and others. In cannabis both pathways could be present, DOXP/MEP pathway for monoterpenes and diterpenes, and MVA pathway for sesquiterpenes and triterpenes. As it was previously mentioned the DOXP/MEP pathway supplies the GPP precursor for the biosynthesis of cannabinoids. There is little knowledge about the regulation of both pathways in the plant cells and which transcriptional factors control them.

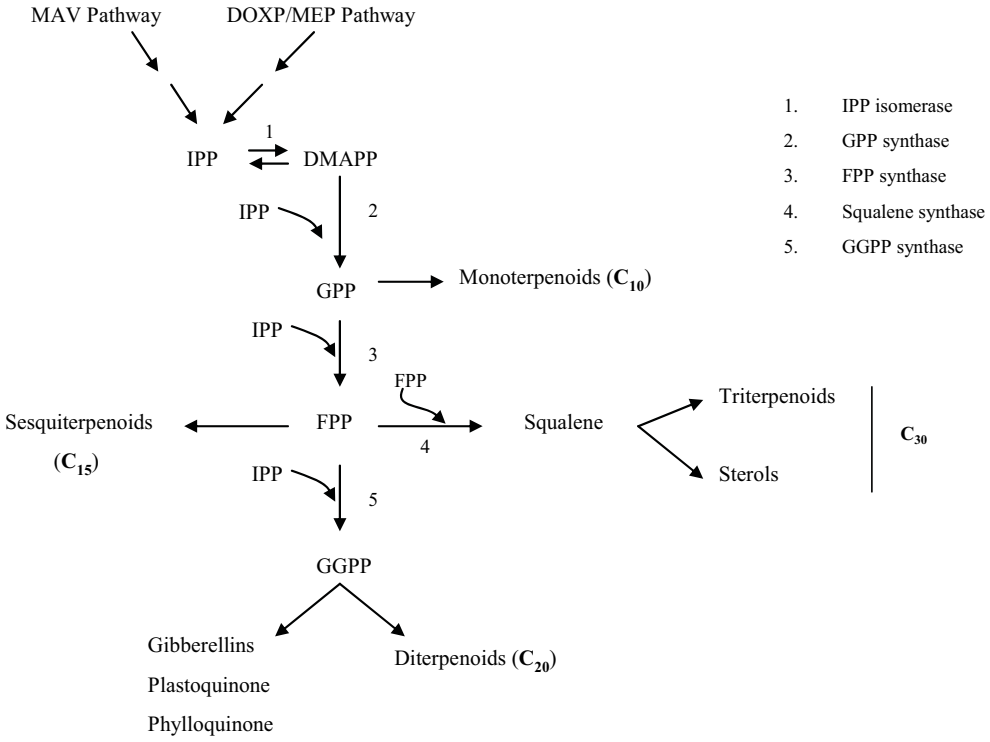


Figure 11. General pathway for the biosynthesis of terpenoids.

I.2.5 Alkaloids

The alkaloids are another major group of secondary metabolites in plants. Alkaloids are basic, nitrogenous compounds usually with a biological activity in

low doses and they can be derived from amino acids. In cannabis 10 alkaloids have been identified (Ross and ElSohly, 1995; Turner *et al.*, 1980). Choline, neurine, L-(+)-isoleucine-betaine and muscarine are protoalkaloids; hordenine is a phenethylamine and trigonelline is a pyridine (Figure 12). Cannabisativine and anhydrocannabisativine are polyamines derived from spermidine and are subclassified as dihydroperiphylline type (Bienz *et al.*, 2002). They are 13-membered cyclic compounds where the polyamine spermidine is attached *via* its terminal *N*-atoms to the β-position and to the carboxyl carbon of a C_{14}-fatty acid (Figure 13). Piperidine and pyrrolidine were also identified in cannabis. These alkaloids have been isolated and identified from roots, leaves, stems, pollen and seeds (El-Feraly and Turner, 1975; ElSohly *et al.*, 1978; Paris *et al.*, 1975). The presence of muscarine in cannabis plants has been questioned (Mechoulam, 1988; ElSohly, 1985).

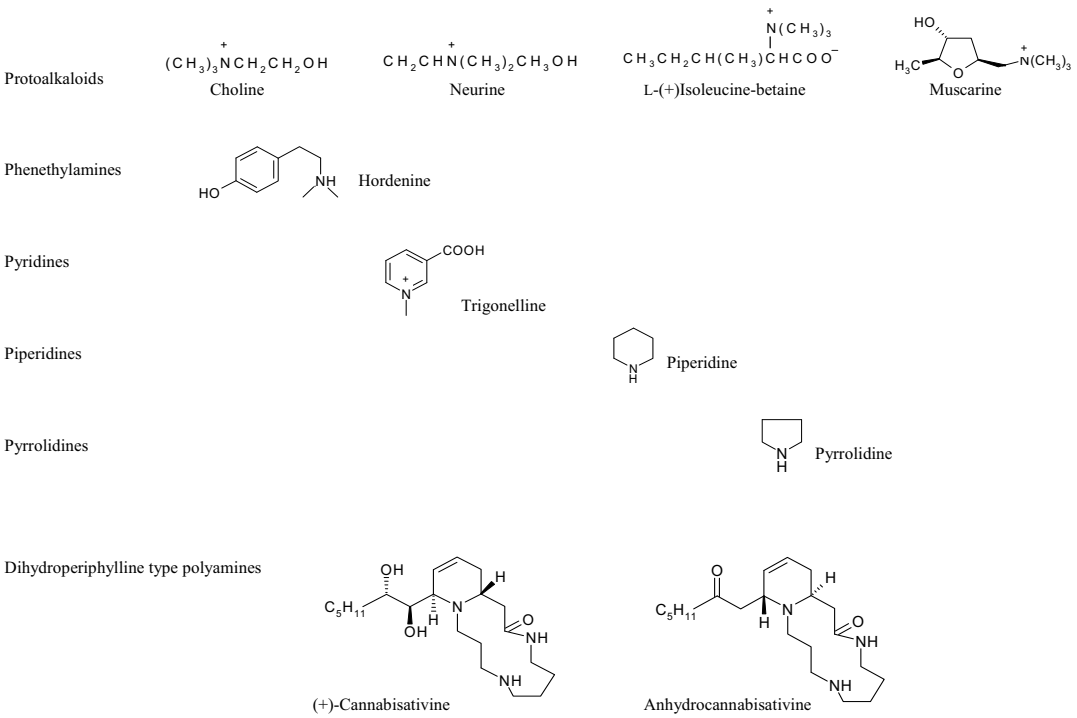


Figure 12. Alkaloids isolated from *C. sativa.*

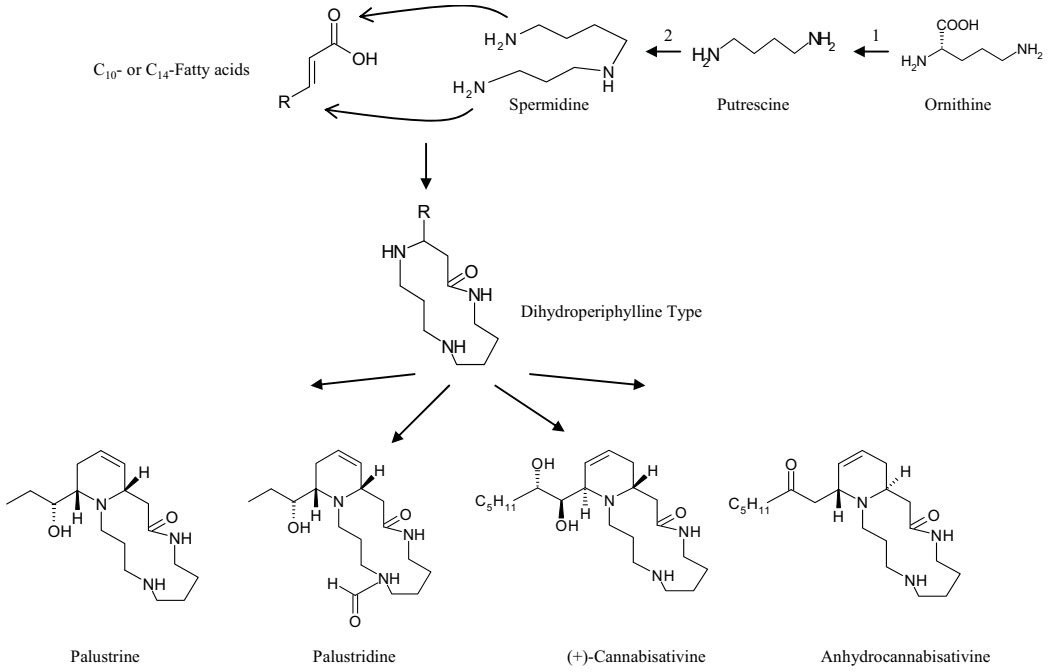


Figure 13. Spermidine alkaloids of the dihydroperiphylline type. 1) Ornithine decarboxylase, 2) Spermidine synthase.

I.2.5.1 Alkaloid biosynthesis

Kabarity *et al*. (1980) reported induction of C-tumors (tumor induced by colchicine) and polyploidy on roots of bulbs from *Allium cepa* by polar fractions from cannabis. It is known that hordenine is a feeding repellent for grasshoppers (Southon and Backingham, 1989) and its presence in cannabis plants could suggest a similar role. The decarboxylation of tyrosine gives tyramine, which on di-*N*-methylation yields hordenine (Brady and Tyler, 1958; Dewick, 2002). Trigonelline is found widely in plants and it has been suggested that it participates in the pyridine nucleotide cycle which supplies the cofactor NAD. Trigonelline is synthesized from the nicotinic acid formed in the pyridine nucleotide cycle (Zheng *et al*., 2004). Choline is an important metabolite in plants because it is the precursor of the membrane phospholipid phosphatidylcholine (Rhodes and Hanson, 1993) and is biosynthesized from ethanolamine, for which the precursor is the amino acid serine (McNeil *et al*., 2000). Piperidine originates from lysine and pyrrolidine from ornithine (Dewick, 2002). The structures of cannabisativine and anhydrocannabisativine are similar

to the alkaloids palustrine and palustridine from several *Equisetum* species (Figure 13). A common initial step in biosynthesis of the ring has been proposed starting with an enantioselective addition of the amine from the spermidine to an α,β-unsatured fatty acid (Schultz *et al.*, 1997). However, there are no studies about the biosynthesis and biological functions of cannabisativine and anhydrocannabisativine. It is known that spermidine is biosynthesized from putrescine, which comes from ornithine (Tabor *et al.*, 1958; Dewick, 2002). In the therapeutic field, Bercht *et al.* (1973) did no find analgesic, hypothermal, rotating rod and toxicity effects on mice by isoleucine betaine. Some other studies suggest pharmacological activities of smoke condensate and aqueous or crude extracts containing cannabis alkaloids (Johnson *et al.*, 1984; Klein and Rapoport, 1971). Due to the low alkaloid concentration in cannabis [the concentration of choline and neurine from dried roots is 0.01% (Turner and Mole, 1973), while THCA from bracts is 4.77% (Kimura and Okamoto, 1970)] chemical synthesis or biosynthesis could be options to have sufficient quantities of pure alkaloids for biological activity testing. New methods for synthesis for cannabisativine (Hamada, 2005; Kuethe and Comins, 2004) as well as the biosynthesis of choline and atropine by hairy root cultures of *C. sativa* (Wahby *et al.*, 2006) have been reported.

I.2.6 Lignanamides and phenolic amides

Cannabis fruits and roots (Sakakibara *et al.*, 1995) have yielded 11 compounds identified as phenolic amides and lignanamides. *N-trans*-coumaroyltyramine, *N-trans*-feruloyltyramine and *N-trans*-caffeoyltyramine are phenolic amides; while cannabisin-A, -B, -C, -D, -E, -F, -G and grossamide are lignanamides (Figure 14). The lignanamides belong to the lignan group (Bruneton, 1999b) and the cannabis lignanamides are classified as lignans of the Arylnaphthalene derivative type (Lewis and Davin, 1999; Ward, 1999).

The phenolic amides have cytotoxic (Chen *et al.*, 2006), anti-inflammatory (Kim *et al.*, 2003), antineoplastic (Ma *et al.*, 2004), cardiovascular (Yusuf *et al.*, 1992) and mild analgesic activity (Slatkin *et al.*, 1971). For the lignanamides grossamide, cannabisin-D and -G a cytotoxic activity was reported (Ma *et al.*, 2002). The presence and accumulation of phenolic amides in response to wounding and UV light suggests a chemical defense against predation in plants (Back *et al.*, 2001; Majak *et al.*, 2003). Furthermore, it has been suggested that

they have a role in the flowering process and the sexual organogenesis, in virus resistance (Martin-Tanguy, 1985; Ponchet *et al.*, 1982), as well as in healing and suberization process (Bernards, 2002; King and Calhoun, 2005). For the lignanamides cannabisin-B and -D a potent feeding deterrent activity was reported (Lajide *et al.*, 1995). It is known that lignans have insecticidal effects (Garcia and Azambuja, 2004).

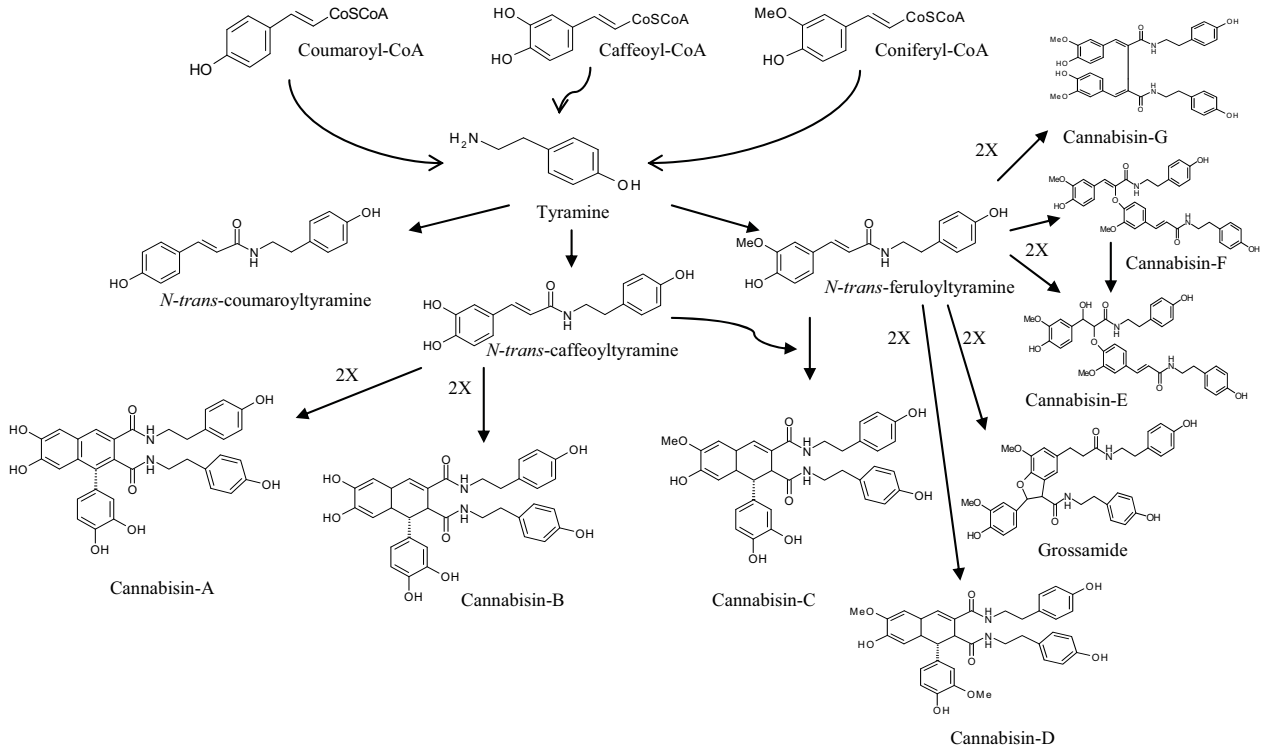


Figure 14. Proposed route for the biosynthesis of phenolic amides and lignanamides in cannabis plants.

I.2.6.1 Lignanamide and phenolic amide biosynthesis

The structures of the lignanamides and phenolic amides from cannabis suggest condensation and polymerization reactions in their biosynthesis starting from the precursors tyramine and CoA-esters of coumaric, caffeic and coniferic acid (Figure 14). It is known that the enzyme Hydroxycinnamoyl-CoA:tyramine hydroxycinnamoyltransferase, E.C. 2.3.1.110 (THT) condenses hydroxycinnamoyl-CoA esters with tyramine (Hohlfeld *et al.*, 1996; Yu and Facchini, 1999). As it was mentioned previously, tyramine comes from tyrosine and the phenylpropanoids from phenylalanine. The amides *N-trans-*

feruloyltyramine and *N-trans*-caffeoyltyramine could be the monomeric intermediates in the biosynthesis of these lignanamides. It has been suggested that these lignanamides could be formed by a random coupling mechanism *in vivo* or they are just isolation artifacts (Ayres and Loike, 1999; Lewis and Davin, 1999); however, biosynthesis studies are necessary to elucidate their origin.

I.3 Conclusion

Cannabis sativa L. not only produces cannabinoids, but also other kinds of secondary metabolites which can be grouped into 5 classes. Little attention has been given to the pharmacology of these compounds. The isolation and identification of the cannabinoids, the identification of the endocannabinoids and their receptors, as well as their metabolism in humans have been extensively studied. However, the biosynthetic pathway of the cannabinoids and its regulation is not completely elucidated in the plant, the same applies for other secondary metabolite groups from cannabis. In three of the mentioned secondary metabolite groups (cannabinoids, flavonoids and stilbenoids), enzymes belonging to the polyketide synthase group could be involved in the biosynthesis of their initial precursors. Only one gene of CHS has so far been identified and more PKS genes are thought to be present for the flavonoid pathway as well as the stilbenoid and cannabinoid pathway. Cannabinoids are unique compounds only found in the cannabis. However, in *Helichrysum umbraculigerum* Less., a species from the family Compositae, the presence of CBGA, CBG and analogous to CBG was reported (Bohlmann and Hoffmann, 1979). Moreover, in liverworts from *Radula* species the isolation of geranylated bibenzyls analogous to CBG was reported (Asakawa *et al*., 1982), suggesting homology of PKS and prenylase genes from the cannabinoid pathway in other species. Crombie *et al*. (1988) reported the chemical synthesis of bibenzyl cannabinoids.

Plants, including *C. sativa*, have developed intricate control mechanisms to be able to induce defense pathways when are required and to regulate secondary metabolite levels in the various tissues at specific stages of their life cycle. Figure 15 shows the currently known various secondary metabolite pathways in cannabis. Research on the secondary metabolism of *C. sativa* as well as its regulation will allow us to control or manipulate the production of the

important metabolites, as well as the biosynthesis of new compounds with potential therapeutic value.

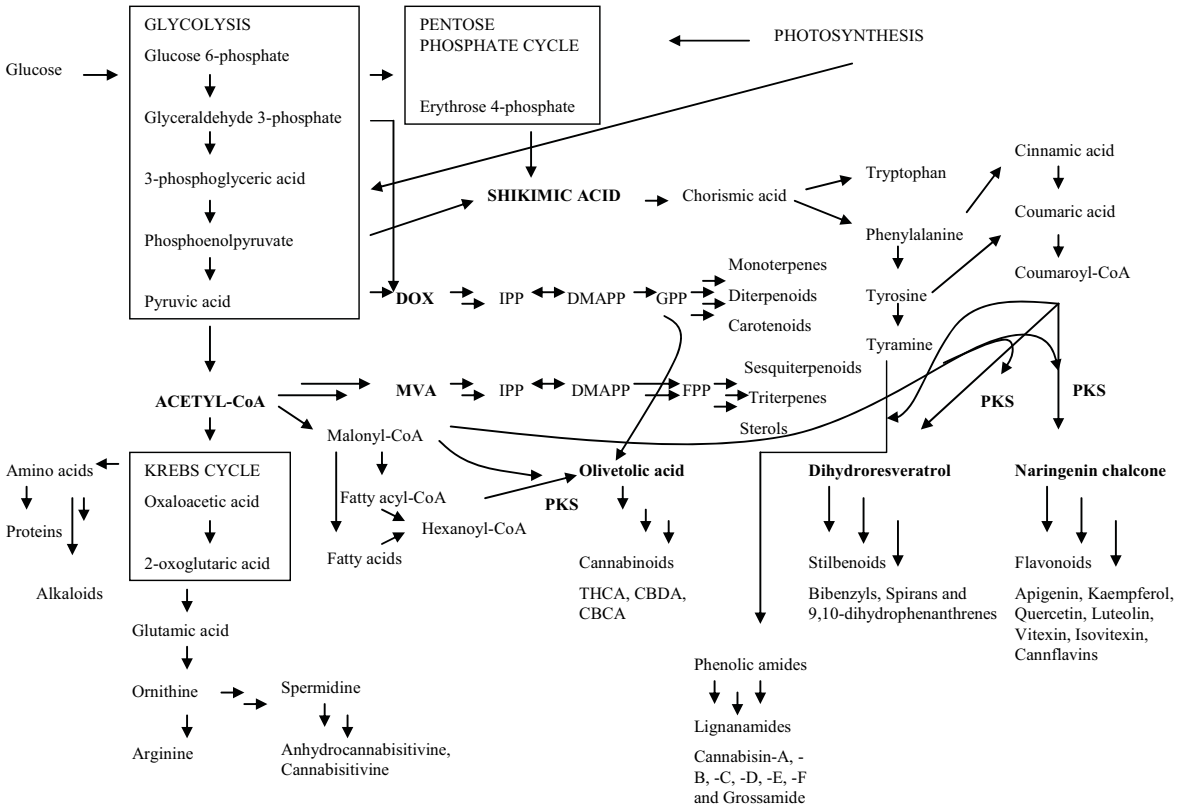


Figure 15. A general scheme of the primary and secondary metabolism in *C. sativa*. For a complete detail of proposed pathways of secondary metabolism see previous figures.

I.4 Outline of the thesis

The studies described in this thesis are focused on biochemical and molecular aspects of PKSs involved in the biosynthesis of precursors from cannabinoid, flavonoid or stilbenoid pathways. A review about general aspects of plant PKS is given in **Chapter 2**. Enzymatic activities of PKSs in plant cannabis tissues and a correlation with the content of cannabinoids and flavonoids is described in **Chapter 3**. Isolation of *PKS* mRNAs and an expression *in silicio* are presented in **Chapter 4**. Finally, as cell cultures can be used as model systems to study secondary metabolite biosynthesis, cannabis cell suspension cultures were treated with biotic and abiotic elicitors to evaluate their effect on the cannabinoid biosynthesis (**Chapter 5**).