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Secondary metabolism in cannabis

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Abstract *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 were 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 disscused.

Keywords Alkaloids · Cannabinoid biosynthesis · Flavones and flavonols · Lignan group · Stilbenes

Introduction

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.

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Linnaeus (1753) considered only one species, *sativa*; however, McPartland et al. (2000) 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 (Wills 1998; Russo 2004; Jiang et al. 2006).

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).

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 (Kushima et al. 1980; Roos et al. 1996; Keller et al. 2001). 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).

Cannabinoids

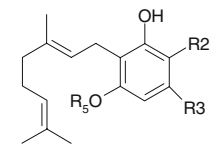
This group represents the most studied compounds from cannabis. The term cannabinoid is given to the terpenophenolic compounds with 22 carbons (or 21 carbons for neutral form) of which 70 have been found so far and which can be divided into 10 main structural types (Fig. 1). All other compounds that do not fit into the main types are grouped as miscellaneous (Fig. 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. Both forms are also further degraded into secondary products by the effects of temperature, light (Lewis and Turner 1978) and autooxidation (Razdan et al. 1972).

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. 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. 1998) and positive effects in psychiatric syndromes, such as depression, anxiety and sleep disorders (Grotenhermen 2002; Musty 2004). These effects could be due to the 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 (Giuffrida et al. 1999; Velasco et al. 2005; Di Marzo et al. 2007). Some therapeutic applications from cannabis, cannabinoids, cannabinoid analogs and CB receptor agonist/antagonist are shown in Table 1.

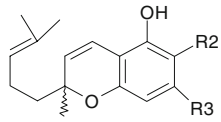
Cannabinoid biosynthesis

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 (Ross et al. 2000; Potter 2004).

The precursors of cannabinoids are synthesized from 2 pathways, the polyketide pathway (Shoyama et al. 1975) and the deoxyxylulose phosphate/methylerythritol phosphate (DOXP/MEP) pathway (Fellermeier et al. 2001) (Fig. 3). From the polyketide pathway, olivetolic acid is derived and from the DOXP/MEP pathway, geranyl diphosphate (GPP) is



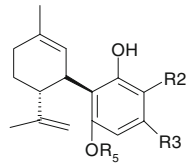
Cannabigerol (CBG) type
R2: H or COOH
R3: C3 or C5 side chain
R5: H or CH_3



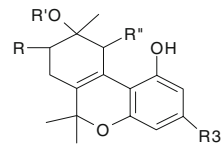
Cannabichromene (CBC) type
R2: H or COOH
R3: C3 or C5

= , *S*-configuration

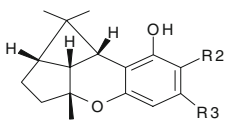
= , *R*-configuration



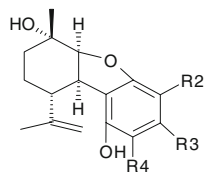
Cannabidiol (CBD) type
R2: H or COOH
R3: C1, C3, C4 or C5 side chain
R5: H or CH_3



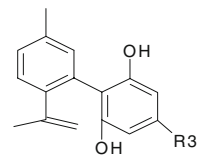
Cannabitriol (CBT) type
R3: C3 or C5 side chain
R: H or OH
R': H or CBDA-C5 ester
R": H, OH or OEt



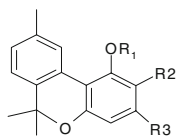
Cannabicyclol (CBL) type
R2: H or COOH
R3: C3 or C5 side chain



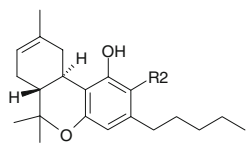
Cannabielsoin (CBE) type
R2: H or COOH
R3: C3 or C5
R4: COOH or H



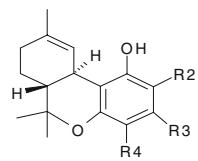
Cannabinodiol (CBND) type
R3: C3 or C5 side chain



Cannabinol (CBN) type
R1: H or CH_3
R2: H or COOH
R3: C1, C2, C3, C4 or C5 side chain



Δ^8-Tetrahydrocannabinol (Δ^8-THC) type
R2: H or COOH

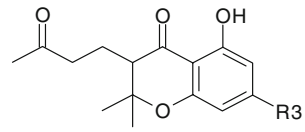


Δ^9-Tetrahydrocannabinol (Δ^9-THC) type
R2 or R4: H or COOH
R3: C1, C3, C4 or C5 side chain
R4: COOH or H

Fig. 1 Cannabinoid structural types

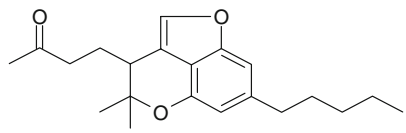
derived. Both are condensed by the prenylase geranyl diphosphate:olivetolate geranyltransferase (GOT) (Fellermeier and Zenk 1998) to form cannabigerolic acid (CBGA), which is a common substrate for three oxydocyclases: Cannabidiolic acid synthase (Taura et al. 1996), Δ^9-Tetrahydrocannabinolic acid synthase (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).

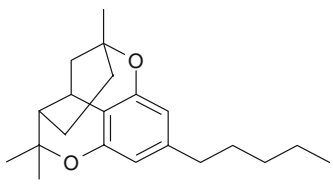


Cannabichromanone

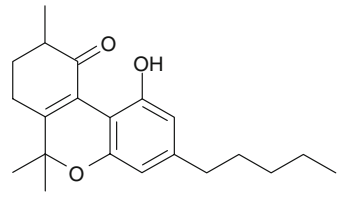
R3: C3 or C5 side chain



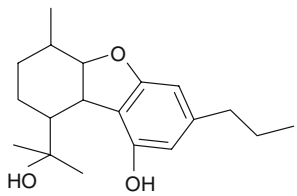
Cannabicoumaronone



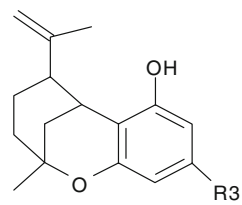
Cannabicitran



10-oxo-$\Delta^{6a(10a)}$-Tetrahydrocannabinol (OTHC)



Cannabiglendol



Δ^7-Isotetrahydrocannabinol

R3: C3 or C5

Fig. 2 Miscellaneous cannabinoids

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 (Uy and Wold 1977; Huber and Hardin 2004). 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. 2007); 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

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[b] 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[a] 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); Henness et al. (2006)/ Sanofi-Aventis; Aronne (2007)

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

[a] 11-OH-Δ^8-THC is primary metabolite from Δ^8-THC, which is further metabolized to [b] Δ^8-THC-11-oic acid by hepatic cytochrome P450s in humans

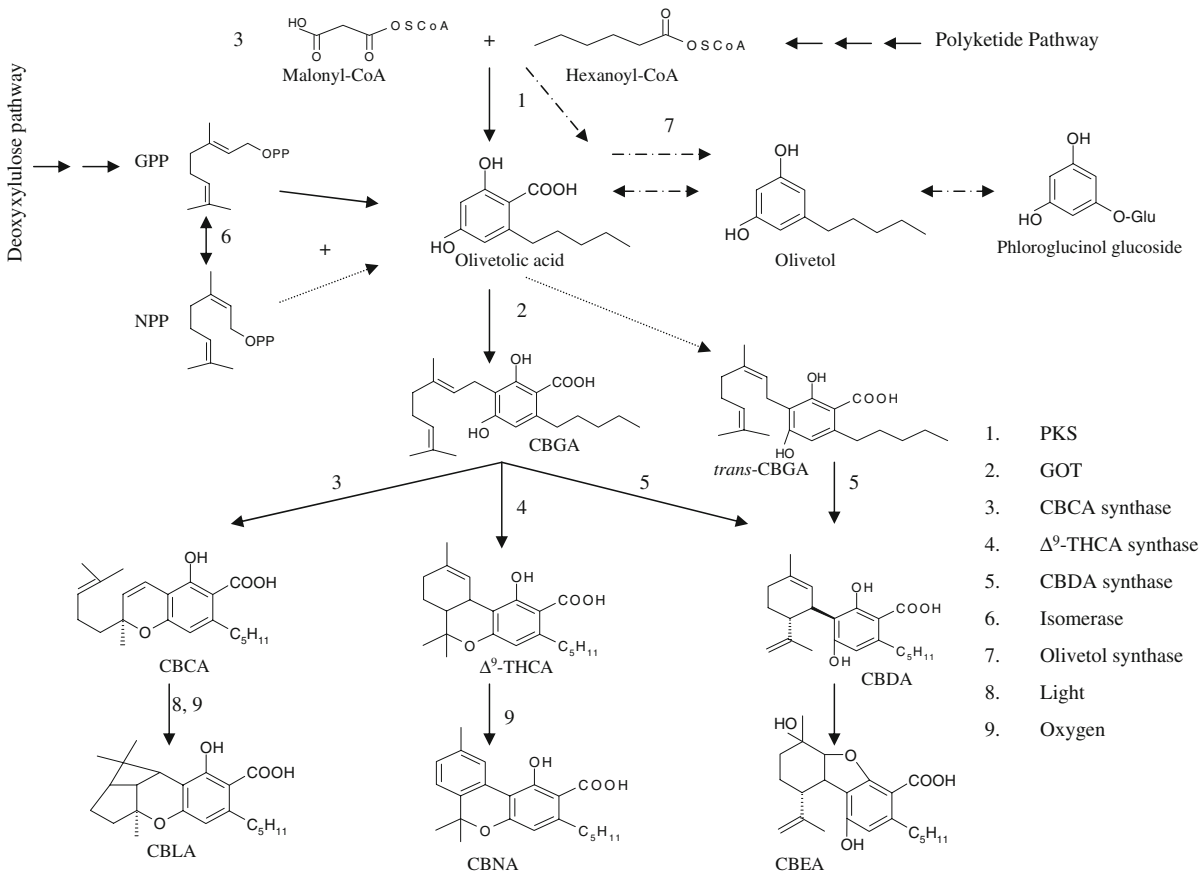


Fig. 3 General overview of biosynthesis of cannabinoids and putative routes

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 (Pastori and del Rio 1997; Jabs 1999; Blokhina et al. 2003). 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 (Turner and ElSohly 1979; Miller et al. 1982) 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

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})	Cofactors	Purity (Sp activity, pKat/mg)	Product	Reference
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	CBDA	Taura et al. (1996)
			206 *trans*-CBGA	5.0		0.39	0.03		Homogeneity (1510)	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)

Mal-CoA, malonyl-CoA; Hex_CoA, hexanoyl-CoA

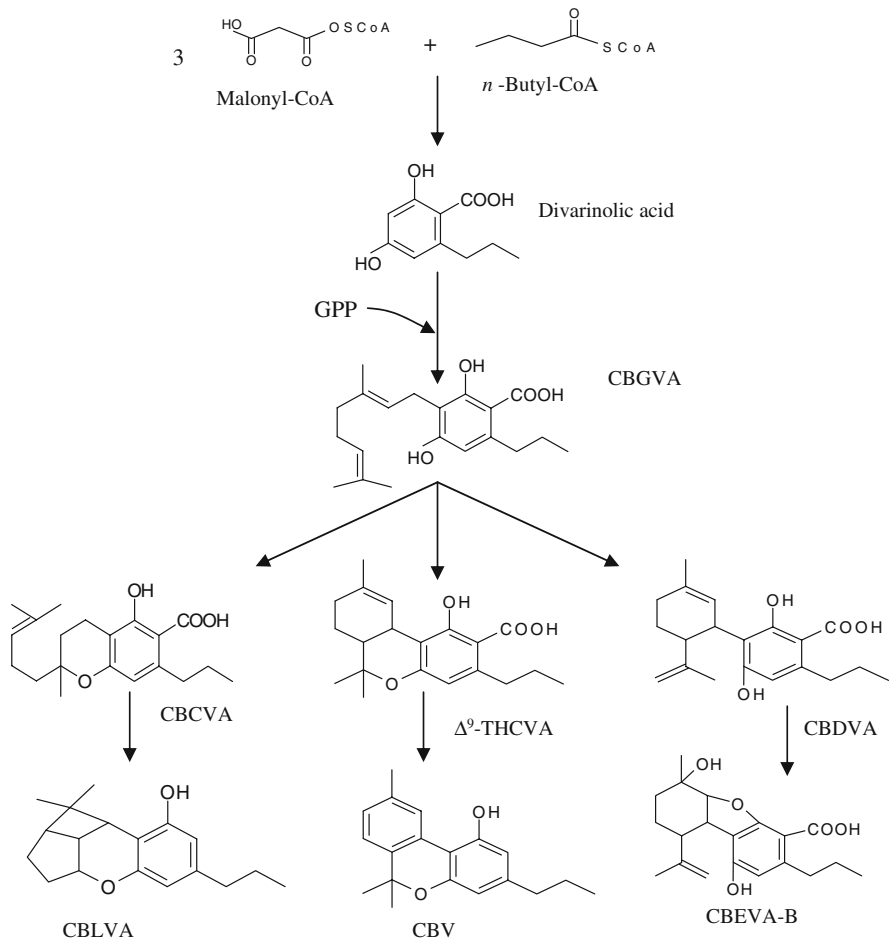


Fig. 4 Proposed biogenetic pathway for cannabinoids with C_3 side-chain

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.

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

Based on the structure of olivetolic acid (Fig. 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 (Fig. 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 suggest 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. 1997) and various oxidized derivatives by microorganisms (Robertson et al. 1978; Binder and Popp 1980) 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).

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 (Fig. 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).

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 Fig. 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

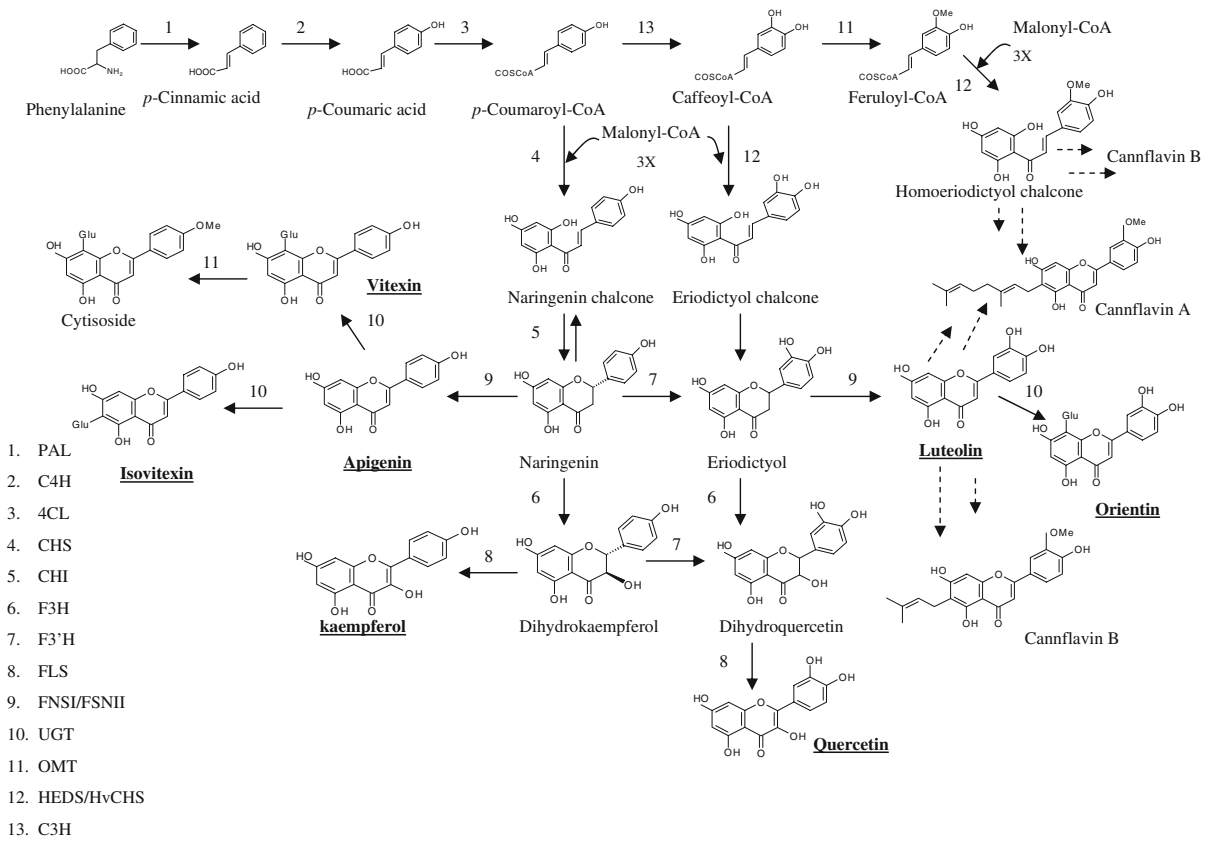


Fig. 5 Proposed general phenylpropanoid and flavonoid biosynthetic pathways in *Cannabis sativa*. C3H, *p-coumaroyl*-CoA 3-hydroxylase; main structures of flavones and flavonols are in bold and underlined

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.

Stilbenoids

The stilbenoids are phenolic compounds distributed throughout wide in the plant kingdom (Gorham et al. 1995). Their functions in plants include constitutive and inducible defense mechanisms (Chiron et al. 2000; 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 (Vastano et al. 2000; Kostecki et al. 2004) or they are repellent towards insects (Hillis and Inoue 1968). Nineteen stilbenoids have been identified in cannabis (Turner et al. 1980; Ross and ElSohly 1995) (Figs. 6, 7, 8).

Although some studies have reported antibacterial activity for some cannabis stilbenoids (Molnar et al. 1986) others have reported that the 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). It has been observed that the stilbenoids show activities such as anti-inflammatory (Adams et al. 2005; Djoko et al. 2007), antineoplastic (Oliver et al. 1994; Iliya et al. 2006; 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).

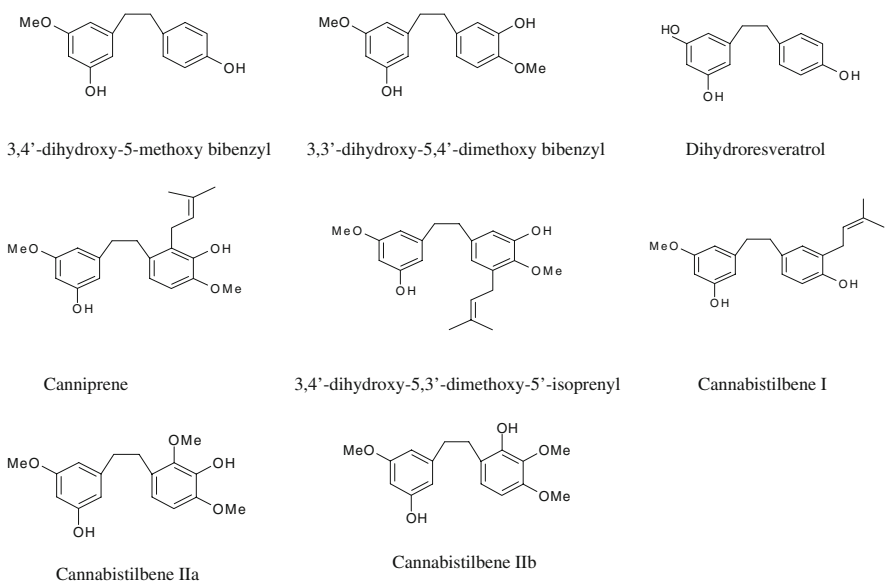


Fig. 6 Bibenzyls compounds in *C. sativa*. The configuration of the structures is not given for simplicity reasons

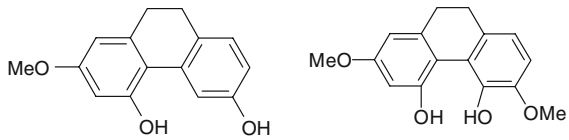


Fig. 7 9,10-dihydrophenanthrenes from *C. sativa*

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).

It has been suggested (Shoyama and Nishioka 1978; Crombie and Crombie 1982) that their biosynthesis could have a common origin (Fig. 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 dihydrocaffeoyl-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 unkown. 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 et al. 1982; Crombie 1986) 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.

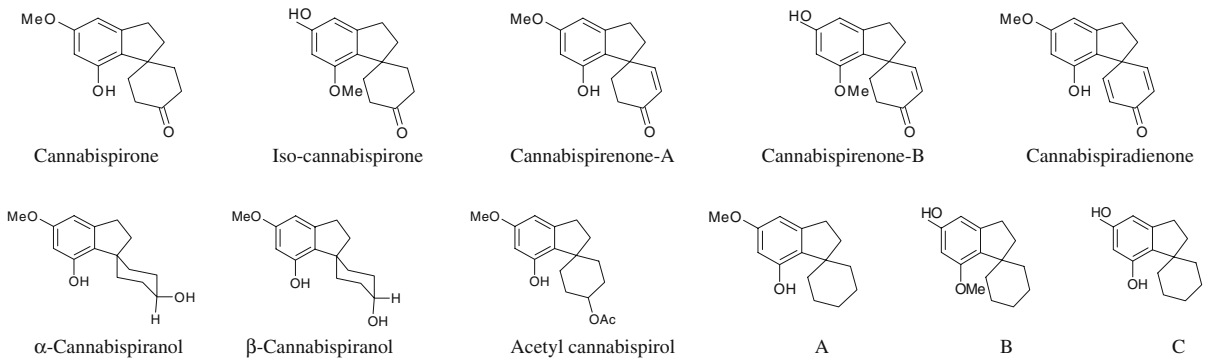


Fig. 8 Spirans from *C. sativa*. A, 7-hydroxy-5-methoxyindan-1-spiro-cyclohexane; B, 5-hydroxy-7-methoxyindan-1-spiro cyclohexane; C, 5,7-dihydroxyindan-1-spiro-cyclohexane

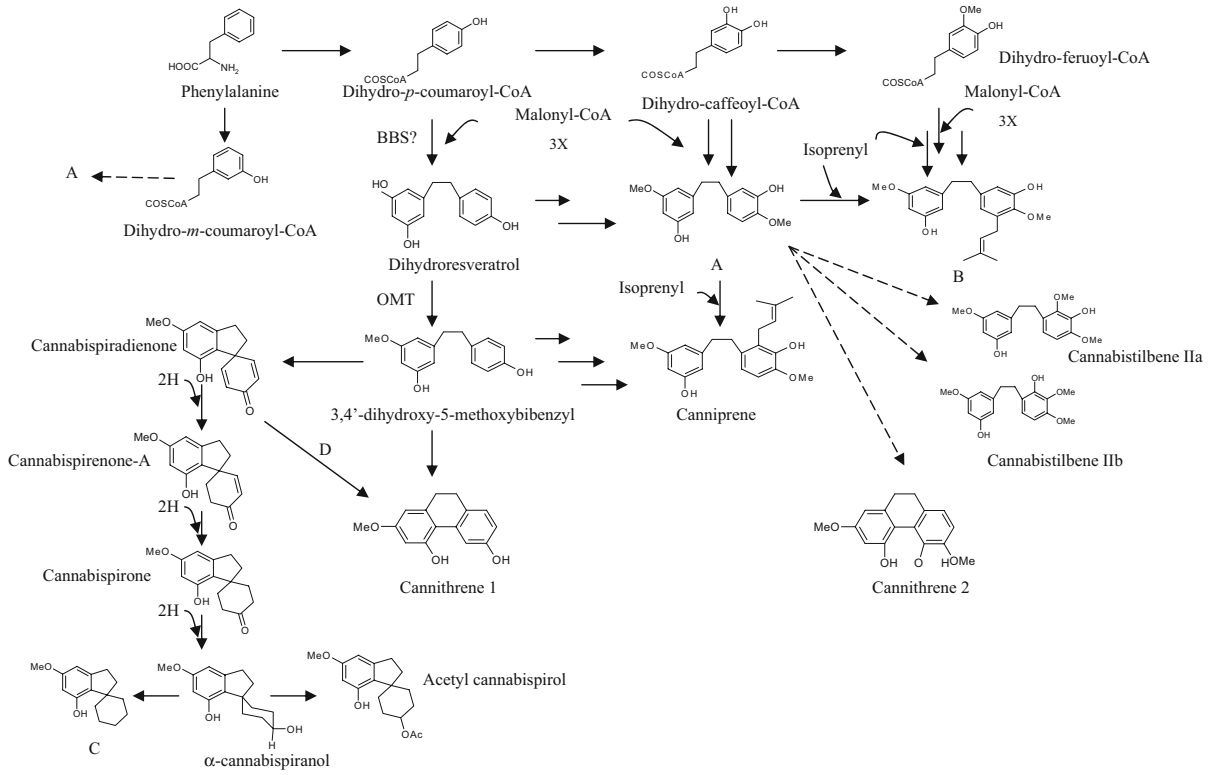


Fig. 9 Proposed pathway for the biosynthesis of stilbenoids in *C. sativa*. (A) 3,3′-dihydroxy-5,4′-dimethoxybibenzyl; (B) 3,4′-dihydroxy-5,3′-dimethoxy-5′-isoprenylbibenzyl; (C) 7-hydroxy-5-methoxyindan-1-spiro-cyclohexane; (D) Dienone-phenol in -vitro rearrangement (heat, acidic pH)

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 (Fig. 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 (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).

Terpenoid biosynthesis

The isoprenoid pathway has been extensively studied in plants (Bouvier et al. 2005). The terpenoids are

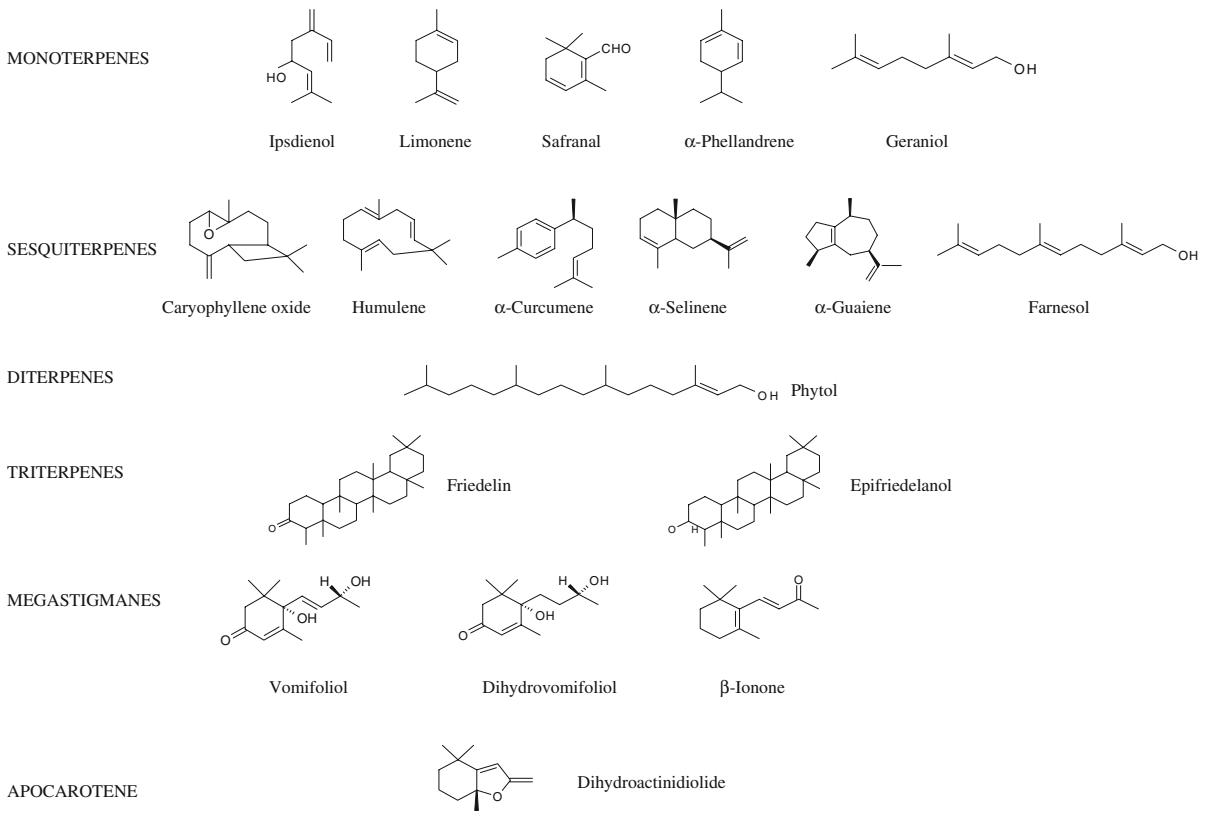


Fig. 10 Some examples of isolated terpenoids from *C. sativa*

derived from the mevalonate (MVA) pathway, which is active in the cytosol, or from the plastidial deoxyxylulose phosphate/methyl-erythritol phosphate (DOXP/MEP) pathway (Fig. 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.

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 (Turner et al. 1980; Ross and ElSohly 1995). Choline, neurine, L-(+)-isoleucine-betaine and muscarine are protoalkaloids; hordenine is a phenethylamine and trigonelline is a pyridine (Fig. 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 (Fig. 13). Piperidine and pyrrolidine were also identified in cannabis. These alkaloids have been isolated and identified from roots, leaves, stems, pollen and seeds (Paris et al. 1975; El-Feraly and

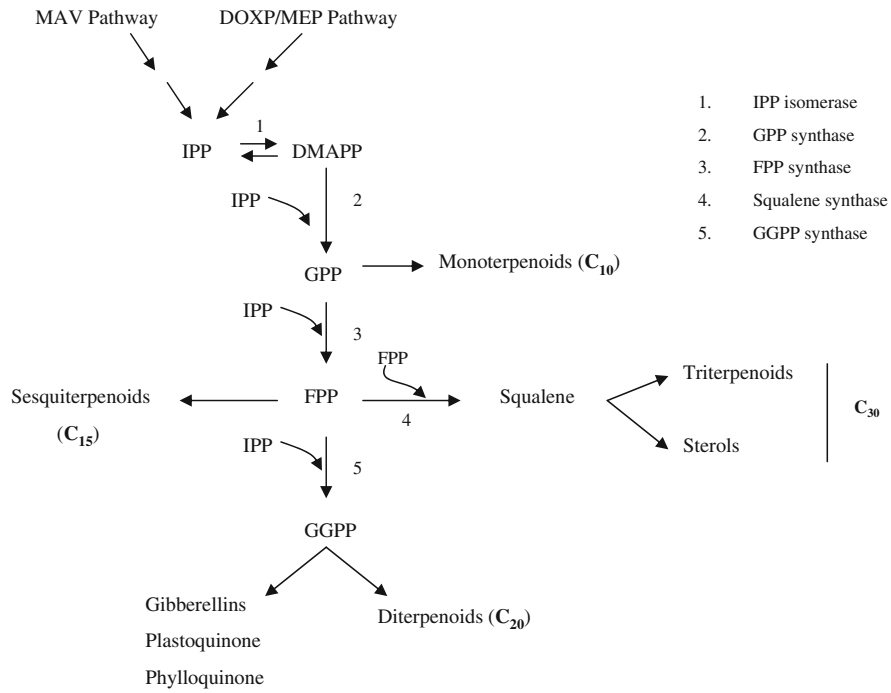


Fig. 11 General pathway for the biosynthesis of terpenoids

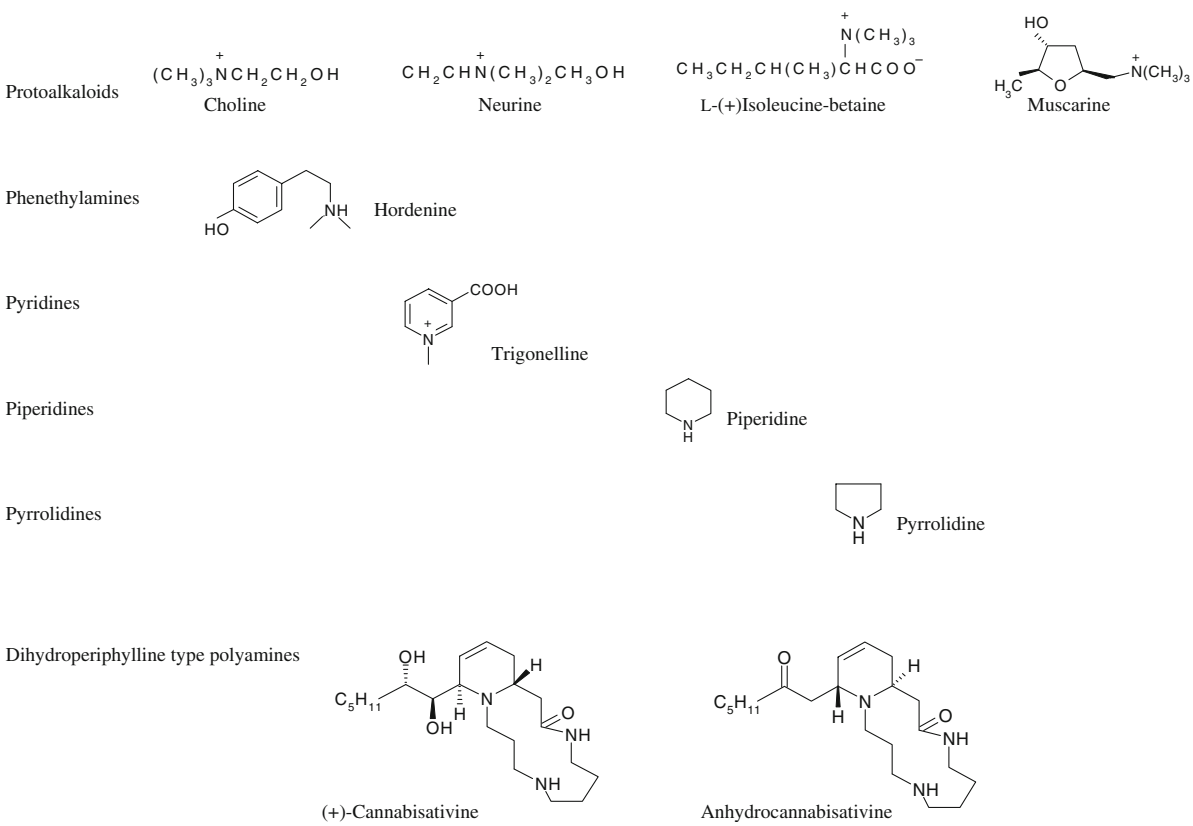


Fig. 12 Alkaloids isolated from *C. sativa*

Turner 1975; ElSohly et al. 1978). The presence of muscarine in cannabis has been questioned (ElSohly 1985; Mechoulam 1988).

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 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 (Fig. 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). 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 (Klein and Rapoport 1971;

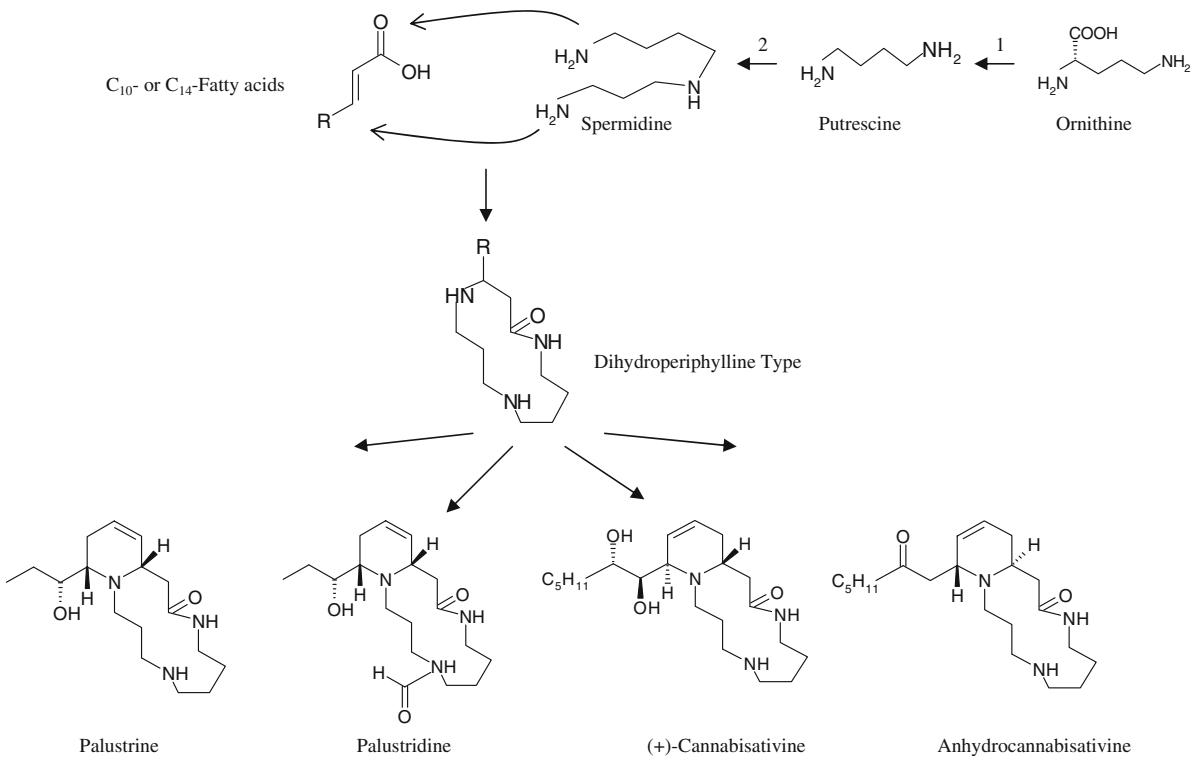


Fig. 13 Spermidine alkaloids of the dihydroperiphylline type. (1) Ornithine decarboxylase, (2) Spermidine synthase

Johnson et al. 1984). 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 (Kuethe and Comins 2004; Hamada 2005) as well as the biosynthesis of choline and atropine by hairy root cultures of *C. sativa* (Wahby et al. 2006) have been reported.

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 (Fig. 14). The lignanamides belong to the lignan group (Bruneton 1999) 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 (Ponchet et al. 1982; Martin-Tanguy 1985), 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).

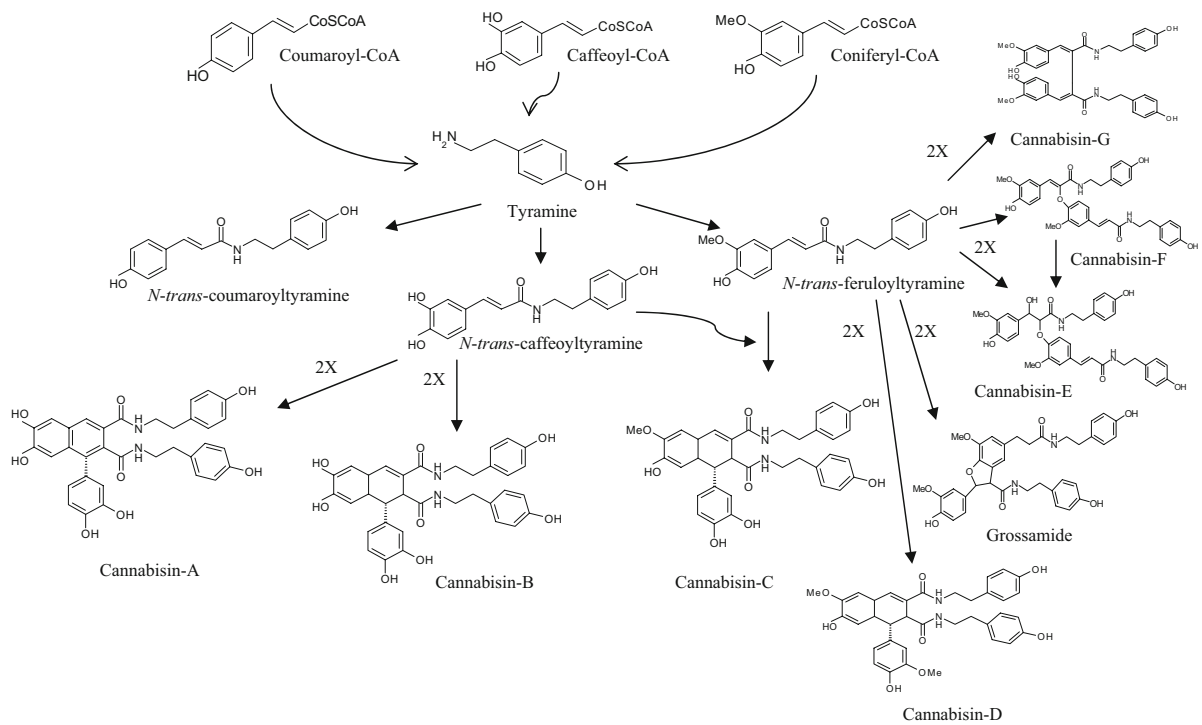


Fig. 14 Proposed route for the biosynthesis of phenolic amides and lignanamides in cannabis plants

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 (Fig. 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 1990; Lewis and Davin 1999); however, biosynthesis studies are necessary to elucidate their origin.

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

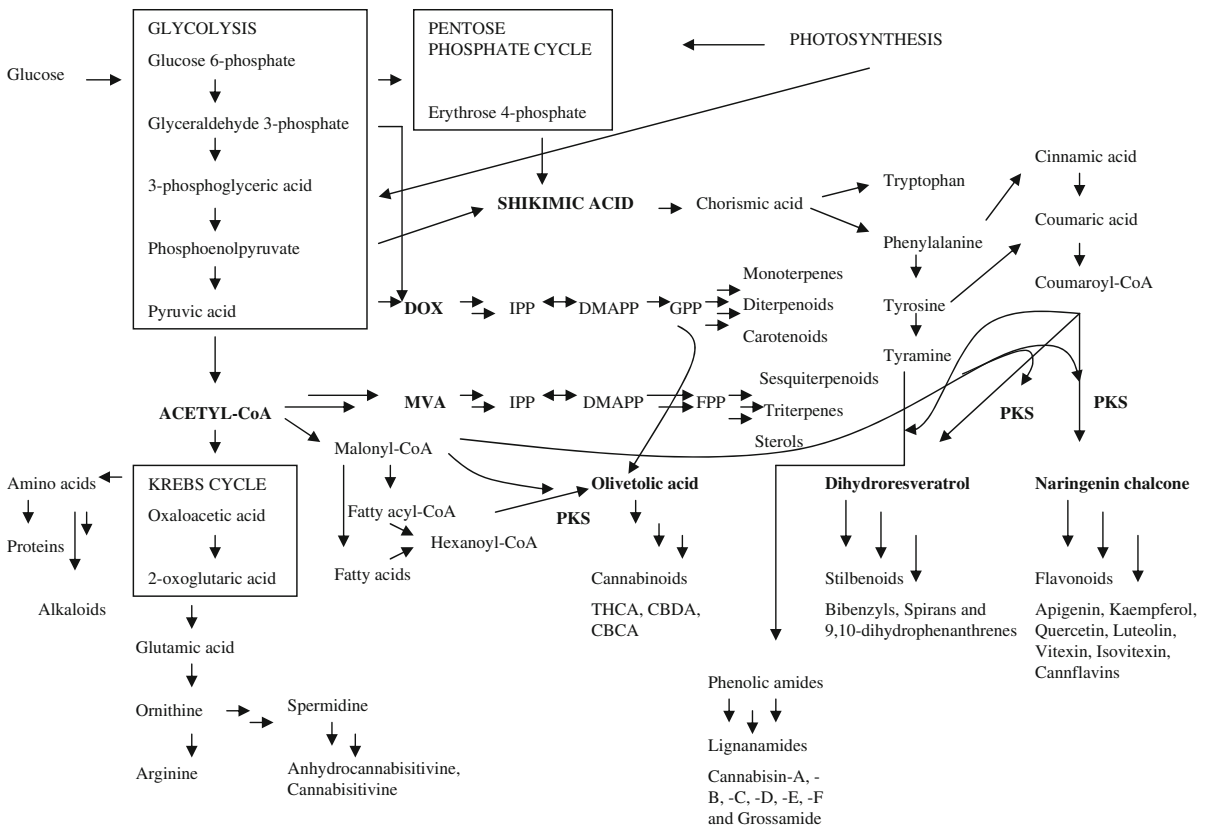


Fig. 15 A general scheme of the primary and secondary metabolism in *C. sativa*. For a complete detail of proposed pathways of secondary metabolism see previous figures

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 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.

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