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Tesi di Laurea:

NOVEL LIGANDS DIRECTLY ENHANCING THE ENDOCANNABINOID SYSTEM: DESIGN AND SYNTHESIS OF NEW *N*-(2-OXO-1,2-DIHYDROPYRIDIN-3-YL)CYCLOHEPTANECARBOXAMIDE DERIVATIVES

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General Introduction

Cannabis sativa L. is an annual dioecious plant, cultivated for over 4500 years. The medical use of Cannabis has a very long history: it has been known for centuries for its therapeutic properties. In 1964, a group of Israeli researchers isolated *trans*-Δ^9-tetrahydrocannabinol (Δ^9-THC), a terpenophenolic compound with psychoactive effects, considered the main constituent of Cannabis plant. Most of biological activities attributed to Cannabis have so far been linked to cannabinoids, and more specifically to THC. In the early 1990s the G-protein coupled cannabinoid receptors (CB) were discovered: cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2).[1] Their ligands are known as cannabinoids or endocannabinoids depending on whether they come from external or internal (endogenous) sources. Nowadays, it is known that many of the body functions are controlled by Cannabis-like substances in brain, immune system and other organs.[2] Since the discovery of the cannabinoid receptors and their endogenous ligands, numerous studies focused on the endocannabinoid system are carried out. Clinical trials into Cannabis, pure cannabinoids and synthetic analogues have demonstrated some effectiveness as analgesics for chronic neuropathic pain, appetite stimulants' for cancer or AIDS patients, and multiple sclerosis. The increased medical interest in these substances has caused the development of various Cannabis-based medicines.[3]

The Botany of Cannabis

Cannabis sativa L. **(Figure 1)** is an annual, usually dioecious, more rarely monoecious, wind-pollinated herb, with male and female flowers developing on separate plants. It propagates from seed, grows vigorously in open sunny environments with well-drained soils, and has an abundant need for nutrients and water. It can reach up to 5 m in height in a 4-6 month growing seasons.

Figure 1. *Illustration of Cannabis sativa L. ("Flora von Deutschland, Österreich und der Schweiz", Otto Wilhelm Thomè).*

The sexes of Cannabis are anatomically indistinguishable before they start flowering, but after that, the development of male and female plants varies greatly. Shorter days, or more accurately longer nights, induce the plant to start flowering: the female plant produces several crowded clusters of individual flowers (flower tops). The male plants finish shedding their pollen and die before the seeds in the female plants ripen.

According to current botanical classification, Cannabis belongs to the family of Cannabinaceae (also Cannabaceae or Cannabidaceae).[2]

Table 1 Current systematic classification of Cannabis[2]

Domain	Eukaryota
Kingdom	Plantae
Superdivision	Spermatophyta
Division	Angiosperms
Class	Dicotyledon
Subclass	Urticales
Family	Cannabinaceae
Genus	*Cannabis*
Species	*Sativa L.*
Varieties	Indica, vulgaris

The upper leaves, unfertilized flower heads, and flower bracts of the female plant are the primary source of cannabinoids in Cannabis. The cannabinoids are enclosed in tiny glandular trichomes found on bracts and floral leaves and unstalked, glandular trichomes found on vegetative leaves and pistillate (flower) bracts and produce the sticky resin containing cannabinoids and terpenes.[4]

Chemical Constituents of Cannabis

With currently 538 known constituents, Cannabis is one of the chemically best-studied plants. But because most of these constituents have not yet been properly characterized for biological activity, the Cannabis plant could be called a *"neglected pharmacological treasure trove"*.

The most important classes of compounds are listed in **Table 2**.

Table 2 An overview of compounds identified in Cannabis[2]

Compound class	Compounds identified
Terpenoids	>120
Cannabinoids	>70
Hydrocarbons	50
Sugars and related compounds	34
Nitrogenous compounds	27
Noncannabinoid phenols	25
Flavoniods	23
Fatty acids	22
Simple acids	21
Amino acids	18
Simple ketones	13
Simple esters and lactones	13
Simple aldehydes	12
Proteins, glycoproteins and enzymes	11
Steroids	11
Elements	9
Simple alcohols	7
Pigments	2
Vitamin	1 (vitamin K)

The most interesting among these constituents are those found in the secretions of the head cells of glandular hairs (trichomes) distributed across the surface of the Cannabis plant. Although trichomes can be found all over the male and female plants, they are particularly concentrated on the bracts that support the female inflorescence. Solitary resin glands, consisting of one or two dozen cells, most often form at the tips of slender trichome stalks that form as extensions of the plant surface. The resin excreted by the glands contains a variety of constituents, any of which might play a role in the biological activities of the Cannabis plant. Among these are terpenoids, flavonoids, and cannabinoids. Resin collects under a thin waxy membrane surrounding the secretory head cells. In these extracellular resin pockets, the secreted compounds are segregated from the secretory cells, protecting it from both oxidative degradation and enzymatic change.[2]

Cannabinoids

Cannabinoids are considered to be the main biologically active constituents of the Cannabis plant, and they can be found nowhere else in nature. The majority of biological activities attributed to Cannabis have so far been linked to cannabinoids, and more specifically to THC. The naturally occurring cannabinoids form a complex group of closely related compounds of which currently 70 are known and well described.

Until the 1980s, the term cannabinoids represented by definition the group of typical terpenophenolic C_{21} compounds present in C. sativa, their carboxylic acids, analogues and transformation products. A modern definition will put more emphasis on synthetic chemistry and on pharmacology, and would also include related structures or compounds that affect cannabinoid receptors. The term *"cannabinoids"* now represents the whole set of endogenous, natural, and synthetic ligands of the cannabinoid receptors, belonging to a wide variety of chemical families.

Chemically, the cannabinoids belong to the terpenophenols, which are common in nature.[2] The most important phytocannabinoid is Δ^9-tetrahydrocannabinol (Δ^9-THC), a terpenophenolic compound with psychoactive effects, considered the main constituent of Cannabis plant **(Figure 2)**.

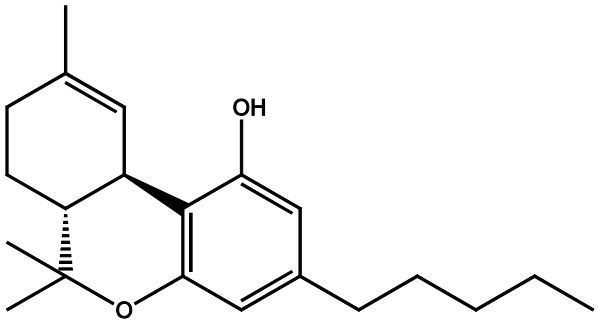


Figure 2. *Δ^9-tetrahydrocannabinol (Δ^9-THC).*

Biosynthesis of Cannabinoids

In all biosynthetic pathways for cannabinoids that were postulated until 1964, cannabidiol (CBD) or cannabidiolic acid (CBDA) was regarded as the key intermediate, which was supposedly built from a monoterpene and olivetol or olivetolic acid (OA), respectively. However, Gaoni and Mechoulam showed that cannabigerol (CBG) is the common precursor of cannabinoids, biosynthesized through the condensation of geranyldiphosphate and olivetol or OA. Subsequently, they concluded that CBD, THC, and cannabinol (CBN) all derive from CBG and differ mainly in the way this precursor is cyclized.

It is now known that cannabinoids are produced by the metabolism of the plant in the form of carboxylic acids, where the substituent at position 2 is a carboxyl moiety (–COOH). The first specific biosynthetic step is the condensation of geranyl pyrophosphate (GPP) with OA into cannabigerolic acid (CBGA), catalyzed by the prenylase enzyme geranyldiphosphate: olivetolate-geranyltransferase (GOT).

Furthermore, biosynthetic pathways finally became clear by identification and subsequent cloning of the genes responsible for the conversion of CBGA to tetrahydrocannabinolic acid (THCA), CBDA, and cannabichromenic acid (CBCA), respectively.

The terpenoid GPP is derived from the deoxyxylulose phosphate/methyl-erythritol phosphate (DOXP/MEP) pathway. Not much is known about the biosynthesis of OA yet, but it has been proposed that a polyketide synthase (PKS) could be involved **(Figure 3)**.[2]

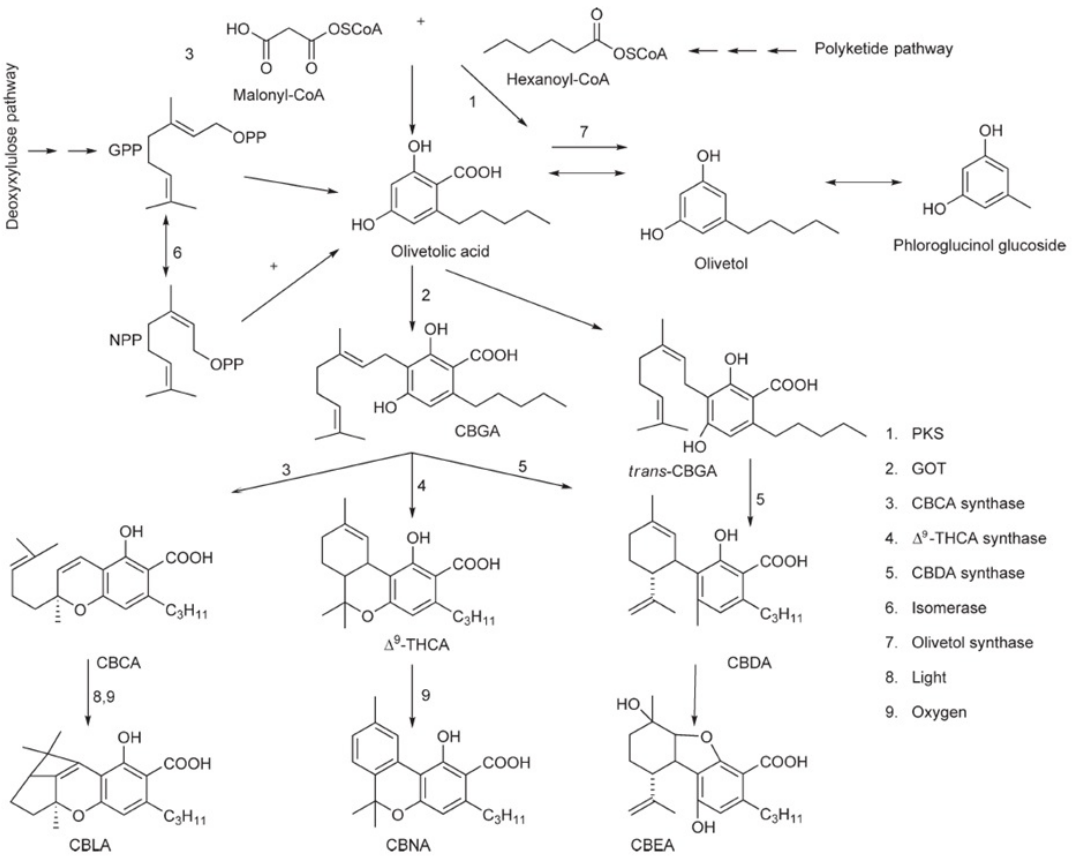


Figure 3. *General overview of the biosynthesis of cannabinoids and putative routes.*[2]

The Endocannabinoid System

The endocannabinoid system is now known to be an ubiquitous neuromodulatory system with wide-ranging actions.[2]

It consists of:

- cannabinoid receptors;
- endogenous cannabinoids (endocannabinoids);
- synthetic and degrading enzymes.[2, 5]

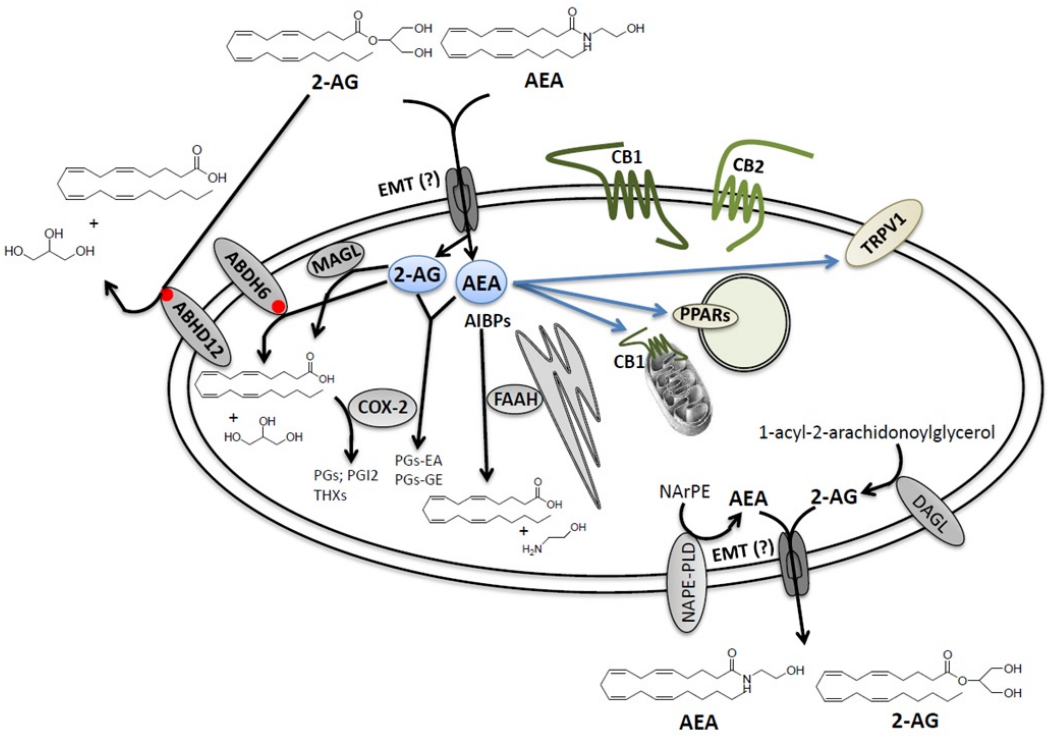


Figure 4. *The Endocannabinoid System: a general overview (International PhD course "Science of Drug and Bioactive Substances", Pisa, (2015), Dr. A. Chicca).*

The Cannabinoid Receptors

Devane *et al.* (1988) first demonstrated the existence of a G-protein-coupled cannabinoid receptor in rat brain that is activated by Δ^9-THC and a gene encoding this protein was identified in 1990 by Matsuda et al. A second G-protein-coupled cannabinoid receptor, that shares sequence similarity with the brain cannabinoid receptor, was identified in immune cells in 1993 and with this discovery the brain receptor was named CB1 (cannabinoid receptor type 1) and the immune cell receptor was named CB2 (cannabinoid receptor type 2).[6, 7, 8]

The human CB1 gene **(Figure 5)** is located on chromosome 6 at locus *q14-q15*. The 472 amino acid residue CB1 receptor protein is encoded by a single exon with 5' and 3' untraslated regions (UTRs).[8, 9]

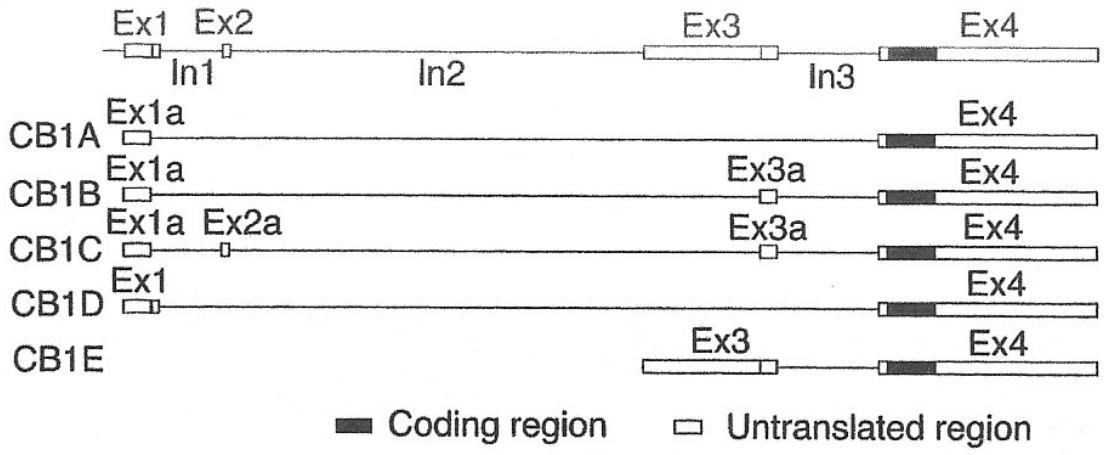


Figure 5. *Human CB1 cannabinoid receptor gene. The diagram shows the structure of the human CB1 gene, comprising three non-coding exons (Ex 1-3), one coding exon (Ex4) and three introns (In 1-3), and alternatively-spliced mRNA transcripts (CB1A, CB1B, CB1C, CB1D, CB1E) that have variable 5' untraslated regions.*[8]

The human CB2 cannabinoid receptor gene is located on chromosome 1 at locus *p36.11* and comprises an exon encoding the 360 amino acid residue receptor protein and a 5' exon encoding an UTR.[8, 9]

The protein sequences of CB1 and CB2 share about 45% identity and 68% similarity for the transmembrane domains.

Cannabinoid receptors belong to the large superfamily of receptors that couple to guanine-nucleotide binding proteins and thread through cell membranes seven times (heptahelical receptors). CB receptors contain a N-terminal extracellular domain that possesses glycosylation sites, a C-terminal intracellular domain coupled to a G-protein complex and 7 hydrophobic transmembrane segments connected by alternating extracellular and intracellular loops **(Figure 6)**.[10]

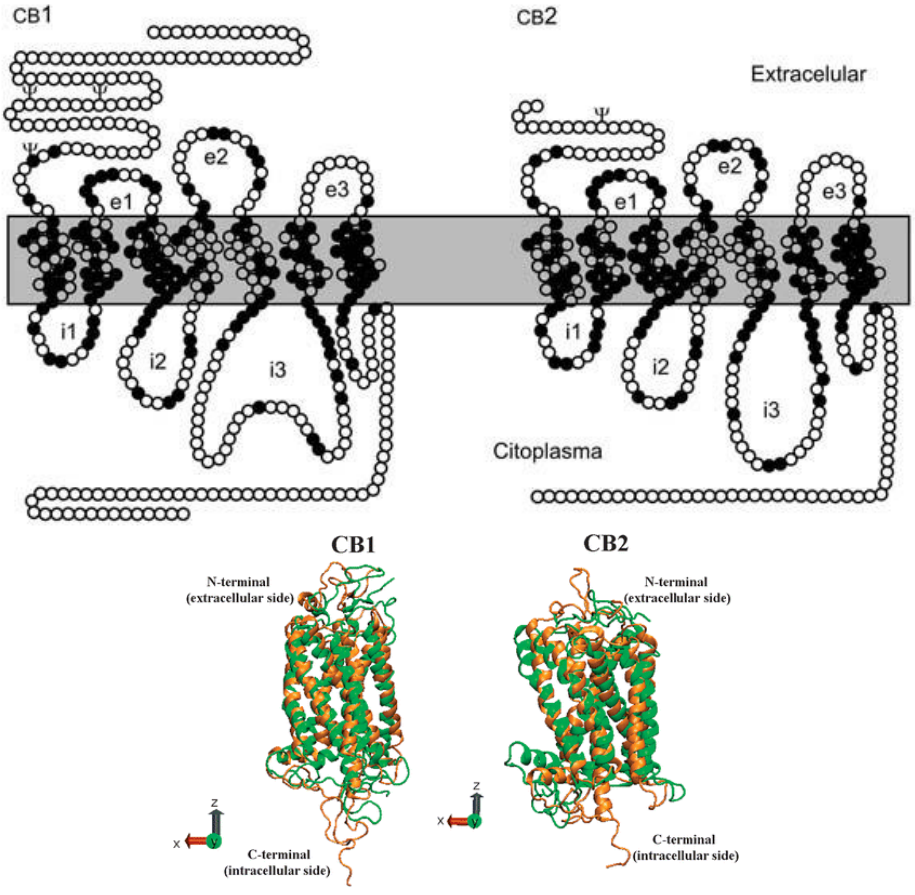


Figure 6. *CB receptors belong to 7TM-GPCRs superfamily.*

The CB1 receptor is most clearly localized in the central nervous system (CNS), therefore it is often called the *"central receptor"*. This receptor is thought to be the most widely expressed G-protein-coupled receptor in the brain: a high density of CB1 receptor was found in the hippocampus, some olfactory regions, caudate,

putamen, accumbens nucleus (ventral striatum), the substantia nigra pars reticulata (SNr), globus pallidus, and the horizontal limb of the diagonal band. CB1 receptor has been also identified in peripheral tissues such as cardiovascular and reproductive cells.[2, 10]

In contrast to previously described predominant presynaptic localisation of CB1 receptor in the brain, CB2 receptor is widely distributed in peripheral tissues and particularly in immune tissues. This receptor was detected also in both microglia and neurons in the CNS.[9, 10]

Both cannabinoid receptors are coupled with G_i or G_o protein, negatively to adenylyl cyclase and positively to mitogen-activated protein (MAP) kinase **(Figure 7)**. CB1 coupling to the G-protein signal transduction pathways in presynaptic nerve terminals transduces the cannabinoid stimulation of MAP kinase and inhibition of adenylyl cyclase, thus attenuating the production of cAMP. CB1 is also coupled to ion channels through $G_{i/o}$ proteins, positively to A-type and inwardly rectifying potassium channels, and negatively to N-type and P/Q-type calcium channels and to D-type potassium channels.

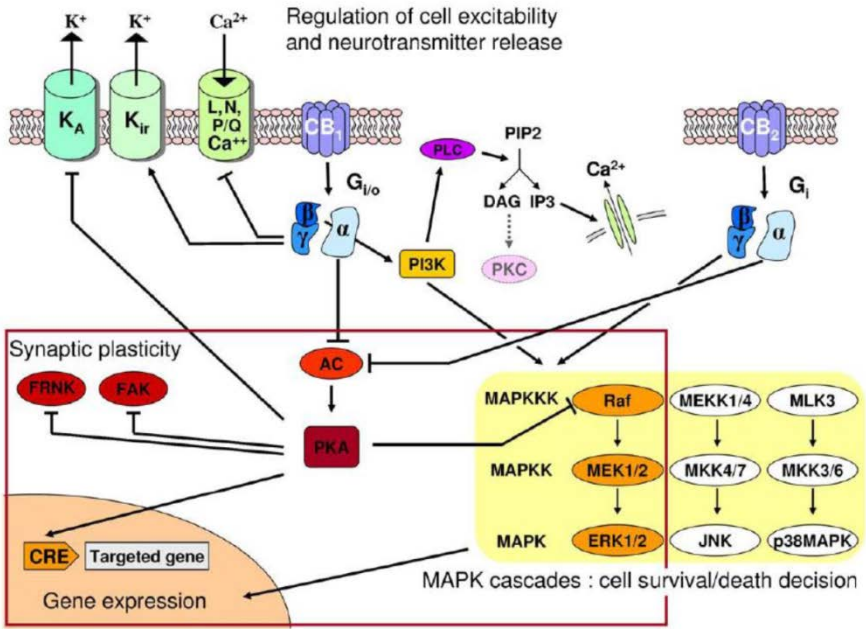


Figure 7. *CB receptors: signal transduction (Boiser B, et al., "Biochem. Pharmacol.", 2010).*

Due to the decrease of cAMP accumulation, cAMP-dependent protein kinase (PKA) is inhibited by CB1 activation. In the absence of cannabinoids, PKA phosphorylates the potassium channel protein, thereby exerting decreased outward potassium current. In the presence of cannabinoids, the phosphorylation of the channel by PKA is reduced, which leads to an enhanced outward potassium current. Based on these findings, it has been suggested that cannabinoids play a role in regulating neurotransmitter releases: inhibition of presynaptic calcium channels reduces neurotransmitter release from CB1-expressing presynaptic terminals.

Presynaptic inhibition of neurotransmitter release by cannabinoids may turn out to be a key neuronal effect of cannabinoids.

It has been proposed that endocannabinoids acting at presynaptic CB1 receptor are responsible for depolarization-induced suppression of excitation or inhibition via the inhibition of Ca^{2+} channel influx.

Endocannabinoids serve as extracellular retrograde messengers, with characteristics very different from other neurotransmitters: they are released by depolarized postsynaptic neurons and may act on the presynaptic CB1 receptor. The resulting inhibition of presynaptic Ca^{2+} channels may serve to limit either excitatory or inhibitory outputs to the postsynaptic neuron from particular terminal: activation of the cannabinoid receptor temporarily reduces the amount of conventional neurotransmitter released (negative feedback mechanism to regulate transmitter release at GABAergic, glutamatergic, and dopaminergic synapses). The ultimate effect of this process depends on the nature of the transmitter that is controlled, which itself depends on the function of the tissue where the cannabinoid receptors are expressed **(Figure 8).**[2, 11, 12]

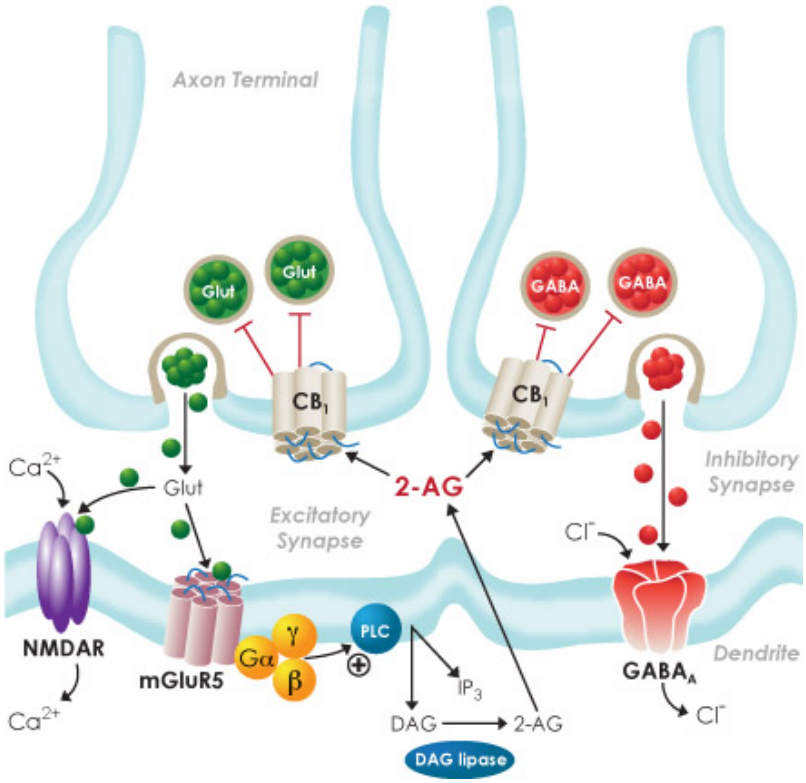


Figure 8. *CB1 activation in the brain: depolarization-induced suppression of excitation or inhibition (Brock T., "Cayman Chemicals").*

The CB2 receptor is also coupled to $G_{i/o}$ proteins and thereby negatively coupled to adenylyl cyclase and the cAMP pathway in various types of cells, and it stimulates mitogen-activated protein kinase (MAPK) cascades. CB2 receptor is located principally in immune cells, among them leucocytes and those of the spleen and tonsils: one of the functions of these receptors in the immune system is modulation of cytokine release. Activation of B- and T-cell CB2 receptor by cannabinoids leads to inhibition of adenylyl cyclase and to a reduced response to immune challenge.[10]

Reward circuitry

The psychoactive properties of marijuana are mediated by the active constituent, Δ^9-THC, interacting primarily with CB1 cannabinoid receptor in a large number of brain areas. However, it is the activation of this receptor located within the central brain reward circuits that is thought to play an important role in sustaining the self-administration of marijuana in humans, and in mediating the anxiolytic and pleasurable effects of the drug.

The reward circuitry of the brain consists of neurons that synaptically connect a wide variety of nuclei. Of these brain regions, the ventral tegmental area (VTA) and the nucleus accumbens (NAc) play central roles in the processing of rewarding environmental stimuli and in drug addiction.[13]

The rewarding properties of addictive drugs are thought to be mediated by their action on the mesolimbic dopamine system. This dopamine system originates in the ventral tegmental area and projects to the ventral striatum, which predominantly comprises the nucleus accumbens. Addictive drugs probably induce their rewarding effects by enhancement of synaptic dopamine levels in the ventral striatum.[14]

The dopaminergic neurons in this circuit do not have CB1 receptor, but they are normally inhibited by GABAergic neurons that do have them. The axon terminals of two GABA containing neurones in the ventral tegmental area might be targets for the action of Δ^9-THC. Both neurones tonically inhibit dopamine-containing neurones and inhibition of GABA release via presynaptic cannabinoid receptors would be expected to increase the activity of the dopaminergic fibre tract **(Figure 9)**.[15]

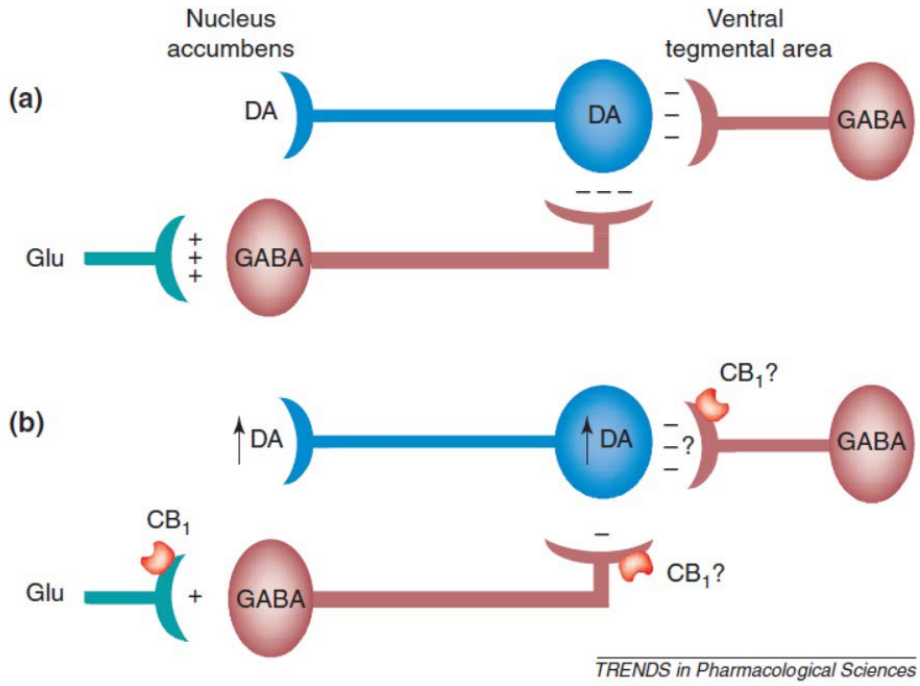


Figure 9. *Effect of Δ^9-THC on the mesolimbic reward system.* ***(a)*** *The dopaminergic fibre tract (blue) projecting from the ventral tegmental area to the nucleus accumbens shell region is under the inhibitory control of a GABA-containing interneurone (red) in the ventral tegmental area and a long-loop GABA-containing feedback neurone (red) projecting from the nucleus accumbens to the ventral tegmental area.* ***(b)*** *Many drugs of abuse increase dopamine release in the nucleus accumbens. The facilitatory effect of Δ^9-THC on dopamine release might theoretically be explained by a direct effect on the dopamine-containing neurone (extremely unlikely) or by activation of presynaptic inhibitory cannabinoid CB1 receptors on the two GABA-containing neurones (more likely). A more plausible explanation would be that Δ^9-THC activates presynaptic inhibitory CB1 receptors on the glutamatergic afferents (green) to the long-loop GABA-containing feedback neurone.*[15]

In 2009 Bossong *et al*. demonstrated the THC-induced dopamine release in the human striatum: cannabinoids induce elevated striatal dopamine levels and these effects are dependent on the activation of cannabinoid CB1 receptor. This effect may be explained by the indirect effects of THC on striatal dopamine levels through cannabinoid CB1 receptor on glutamate and GABA neurons in the nucleus accumbens and the ventral tegmental area.[14]

Endogenous Cannabinoids

Endocannabinoids are so named because they were first identified as activating the same receptors as cannabinoids, the primary psychoactive components of Cannabis.[2, 5, 10]

The discovery of the presence of receptors capable of binding THC, led the researchers to hypothesize the existence of an endogenous ligand able to bind cannabinoid receptors. In fact, in 1992, Devane *et al*. isolated, from the brain tissue of pigs, the first endogenous ligand capable of a selective binding to cannabinoid receptor (CB1): *N*-arachidonoylethanolamine or anandamide (AEA). Chemically, AEA is an amide resulting from the condensation between arachidonic acid and ethanolamine **(Figure 10)**.

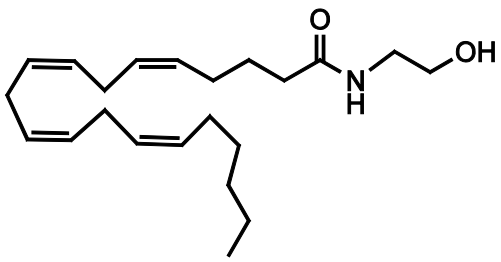


Figure 10. *Structure of anandamide (AEA).*

A few years later, another related ligand, belonging to the class of monoacylglycerol, was discovered from canine gut: 2-arachidonylglycerol (2-AG) **(Figure 11)**. This lipid was present at a concentration 100-200 times higher than AEA and had the same effects of THC. 2-AG was proposed as the main CB2 ligand. Later it was discovered that while AEA preferentially activated the CB1, 2-AG could activate indifferently both types of cannabinoid receptors.

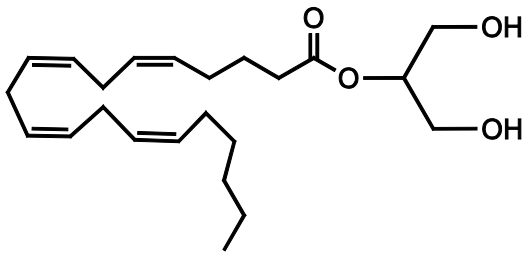


Figure 11. *Structure of 2-arachidonylglycerol (2-AG).*

Currently, several derivatives of fatty acid and analogues of AEA and 2-AG are known: *N*-arachidonoyl dopamine (NADA), 2-arachidonyl glyceryl ether (Noladin ether), able to bind both cannabinoid receptors, and virodhamine, with an antagonist activity towards CB1 receptor.[1]

Synthetic pathway

A feature that distinguishes endocannabinoids from many other neuromodulators is that they are not synthesised in advance and stored in vesicles. Rather, their precursors exist intracellularly in the plasma membrane of neurons as part of certain phospholipids and are cleaved by specific enzymes. This form of synthesis is often referred to as *"on demand"*: endocannabinoids are produced by distinct biochemical pathways involving phospholipases C and D, as well as other enzymes. These events are triggered by the enhancement of intracellular calcium concentrations that follow cell depolarization or the mobilization of intracellular calcium stores subsequent to the stimulation of protein-coupled receptors from the G_q/G_{11} family. Accordingly, the enzymes catalyzing anandamide and 2-AG are calcium-sensitive.[2, 5, 10]

The mechanisms of anandamide biosynthesis in the nervous system are incompletely understood. The most accepted pathway for the synthesis of AEA involves the action of Ca^{2+}-dependent *N*-acyltransferase (NAT) and a specific lipase called *N*-acylphosphatidylethanolamine phospholipase D (NAPE-PLD).

NAT transfers the arachidonic acid from the phosphatidylcholine *sn*-1 position to the primary amine of phosphatidylethanolamine to form *N*-arachidonoyl phosphatidylethanolamine (NArPE). NAPE-PLD hydrolyzes NArPE, in presence of Ca^{2+}, to release AEA and phosphatidic acid.

Multiple mechanisms and putative anandamide biosynthetic enzymes have been suggested. A second synthetic pathway involves the hydrolysis of NArPE (produced by NAT) to phospho-AEA (p-AEA), catalyzed by phospholipase C (PLC). Afterwards, p-AEA is dephosphorylated to AEA **(Figure 12A)**.[1, 16]

2-AG is also synthesized *"on demand"* from membrane phospholipid precursors.

2-AG is synthesized from arachidonoyl-containing diacylglycerol (DAG) species by *sn-1*-specific diacylglycerol lipase-α and -β (DAGLα and DAGLβ): 1-acyl-2-arachidonylglycerol (DAG) is the first product of this synthetic pathway.

DAG could be synthesized through two different reactions: one catalyzed by PLC, which operates on phosphatidylinositol, and the other by a phosphohydrolase, which hydrolyzes phosphatidic acid. Diacylglycerol lipase (DAGL) turns DAG into 2-AG. PLC could also turn phosphatidylinositol (PI) into *lyso*-PI and it is transformed in 2-AG by a Ca^{2+}-dependent PLC **(Figure 12B)**.[1, 16]

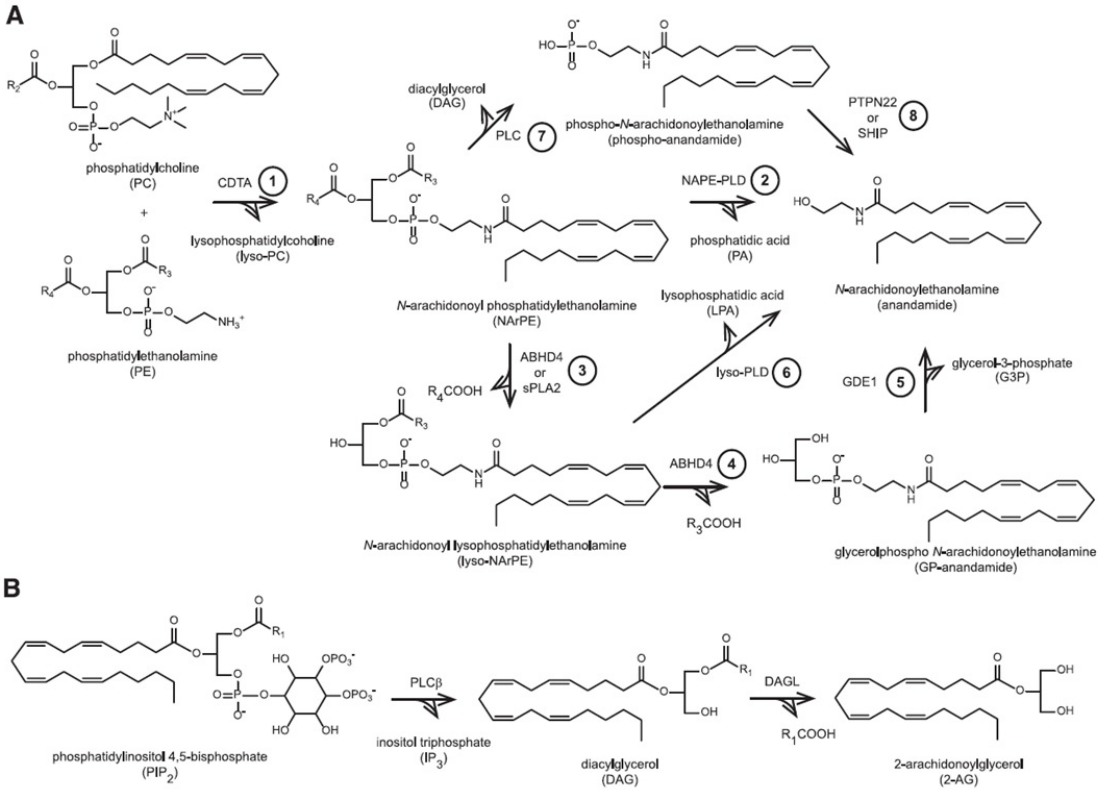


Figure 12. ***(A)*** *Anandamide biosynthesis* ***(B)*** *2-AG biosynthesis.*[16]

Transport mechanism

After formation, endocannabinoids are transported across the cell membrane to interact with their extracellular binding sites on cannabinoid receptors.[2]

Endocannabinoids, because of their lipophilic nature, are able to cross the lipid bilayer. Nevertheless, researchers think about the existence of a specific carrier on the plasma membrane that internalizes endocannabinoids. Although this protein has not been identified yet, its activity has been widely featured in many neuronal and peripheral cells, in peripheral organs and central nervous system (CNS).

It has been postulated the existence of specific membrane carrier of endocannabinoids: endocannabinoid membrane transporter (EMT). EMT let endocannabinoids to cross the plasma membrane. The diffusion occurs in both directions (in and out of the membrane) and does not depend on ATP hydrolysis.[1]

Degrading enzymes

Endocannabinods' biological action has a short life, depending on a deactivation process: reuptake, enzymatic hydrolysis and riesterification of products.

In the nervous system, anandamide and 2-AG are degraded primarily by the serine hydrolase enzymes fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively.[16]

Anandamide is extremely unstable, and quickly hydrolysed by FAAH yielding ethanolamine and arachidonic acid **(Figure 13)**. Two mechanisms for anandamide inactivation have been identified in the brain. The first is intracellular hydrolysis by FAAH.[1, 10]

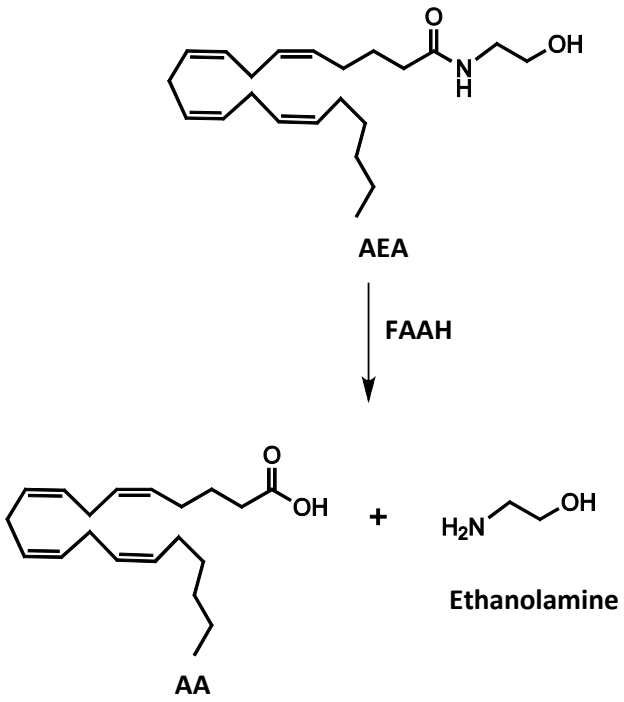


Figure 13. *Anandamide degradation.*[1]

FAAH is both a serine hydrolase, an enzyme class that utilizes a nucleophilic serine for catalysis, and a member of the amidase signature enzyme family. FAAH is an ~60 kDa integral membrane protein that is highly expressed in the mammalian brain where it localizes to intracellular membranes of postsynaptic somata and dendrites. It is a homodimeric enzyme that can accommodate its substrate into a complex architecture of the catalytic site, which is characterized by three binding

channels. Substrates are thought to reach the catalytic site via a membrane access (MA) channel where two charged residues (Asp_{403} and Arg_{486}) may favour the entrance of the polar head groups of fatty acid molecules. The catalytic action of FAAH occurs in the core of the binding site where an unusual catalytic triad (Ser_{241}–Ser_{217}–Lys_{142}) performs the hydrolysis of the substrate, while an oxyanion hole keeps the substrate properly oriented for hydrolysis. Tightly connected to the catalytic region, a cytosolic port (CP) allows the exit of the leaving group after substrate hydrolysis. A third acyl-chain binding (AB) cavity, adjacent to the MA channel, seems to contribute to the proper accommodation of the substrate during catalysis **(Figure 14)**.[16]

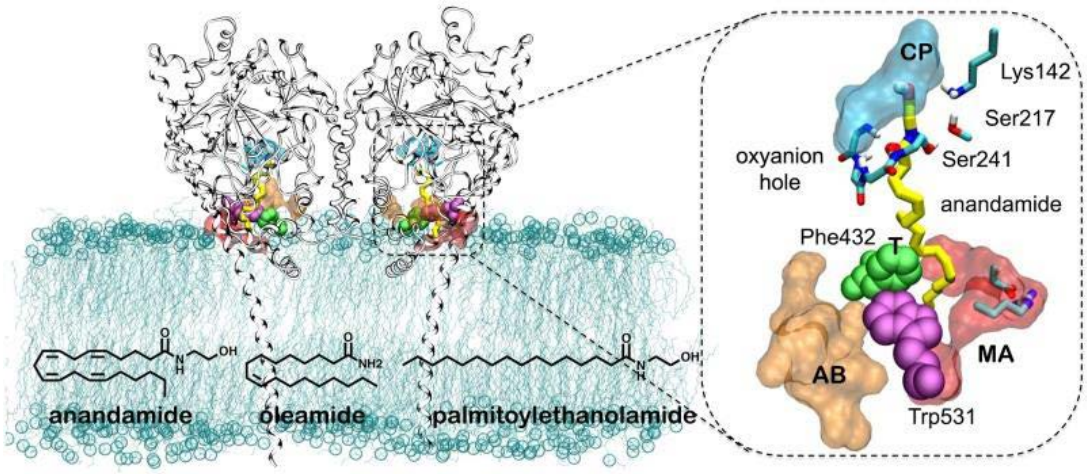


Figure 14. *FAAH catalytic activity (Palermo G. et al., "J Chem Theory Comput", 9, 2013, pp. 1202-1213).*

After the discovery of 2-AG as a second endocannabinoid, its inactivation in the nervous system was hypothesized to proceed through monoacylglycerol lipase (MAGL), a soluble serine hydrolase that peripherally associates with cell membranes (inner plasma leaflet).[16] In the CNS is highly expressed in the pre-synaptic neurons (colocalized with CB1 receptor in the axon terminals), astrocytes and glial cells. In the periphery is abundantly expressed in many tissue and immune cells (low or negligible in macrophages).[17] MAGL hydrolyzes the ester bond of 2-AG, releasing arachidonic acid and glycerol (**Figure 15**).[1]

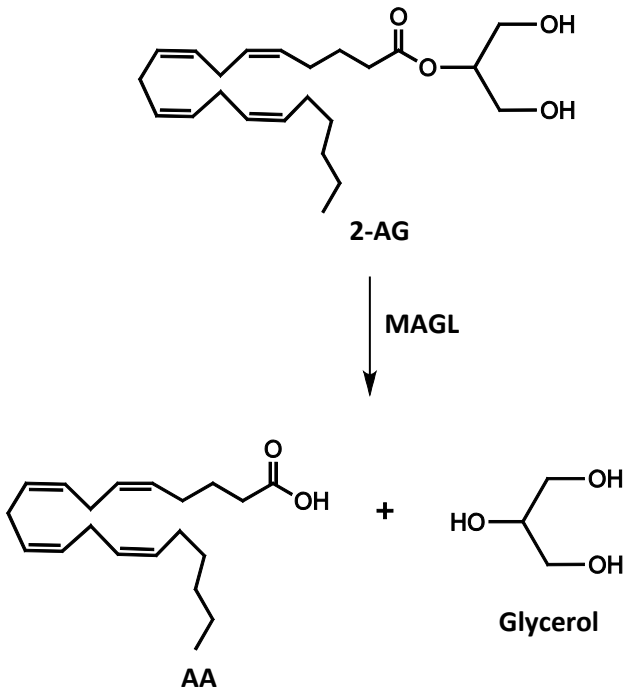


Figure 15. *2-AG degradation.*[1]

There is evidence that, in addition to MAGL, the brain expresses other enzymes that can hydrolyze 2-AG: the postgenomic proteins α/β-hydrolase domain containing ABHD6 and ABHD12 that remain poorly characterized.

ABHD6 is an integral membrane protein localizing to the post-synaptic neuronal membrane and in the glia. The active site is predicted to face the cytosol/intracellular membrane. In neurones ABHD6 localizes to sites of 2-AG generation, including post-synaptic dendrites of principal glutamatergic neurones as well as some GABAergic interneurones.

ABHD12 is predicted to be an integral membrane protein highly expressed in microglia. ABHD12 transcripts are also found in related cell types (macrophages, osteoclasts) and this hydrolase is therefore potentially positioned to guard 2-AG-CB receptor signaling in these cells. The active site is predicted to face the luminal/extracellular side.[18, 19]

Therapeutic potential of Cannabinoids

Cannabinoids are best known for their effects on CNS functions.

They produce euphoria, alterations in cognition and analgesia, have anticonvulsivant properties and affect temperature regulation, sleep and appetite. However, cannabinoids also possess immunomodulatory activity and anti-inflammatory properties. Many disease of the CNS including Alzheimer disease, Parkinson's disease, AIDS dementia and mainly Multiple Sclerosis (MS) involve inflammation, and cause an upregulation of cytochines and other inflammatory mediators in the CNS. Therefore cannabinoids may be potential therapeutic agents in neurological diseases.

Role of CB receptors in Neuroprotection

Accumulated evidence has suggested a role of the CB receptors in neuroprotection. The blood-brain barrier (BBB) is an important brain structure that is essential for neuroprotection. A link between the CB receptors and the BBB is thus likely, but this possible connection has only recently gained attention. Cannabinoids and the BBB share the same mechanisms of neuroprotection and both protect against excitotoxicity (CB1), cell death (CB1), inflammation (CB2) and oxidative stress (possibly CB independent), all processes that also damage the BBB. The CB receptors were shown to improve BBB integrity, particularly by restoring the tightness of the tight junctions. Both CB receptors are able to restore the BBB and neuroprotection, but much uncertainty about the underlying signaling cascades still exists.

The most extensively studied mechanism of neuroprotection includes the anti-inflammatory effects of the CB2 receptor, in which CB2 protects the brain by preventing neuroinflammation. In contrast, the CB1 receptor has been implicated in protection against cell death induced by an overstimulation of excitatory

receptors and concurrent calcium release, also known as excitotoxicity **(Figure 16)**.[20, 21]

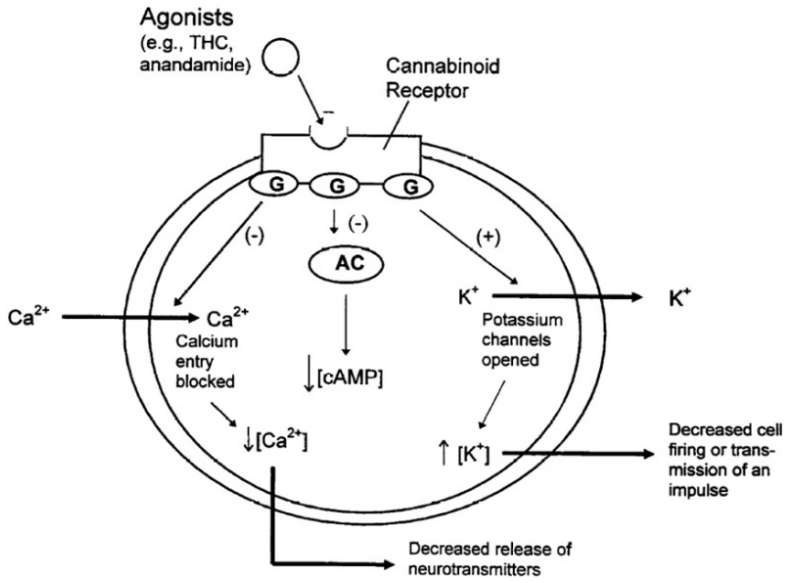


Figure 16. *The influence of the CB1 receptor on intracellular calcium levels and neurotransmitter release as is relevant in excitotoxicity.*[20]

Role of CB receptors in Neuropathogenesis

The CB1 and CB2 have been implicated in a number of neuropathogenic processes. The cannabinoid receptor type that is linked to an inflammatory response within the CNS appears to be CB2. Microglia apparently act as the major expressers of this receptor during early stages of neuroinflammation. As resident macrophages in the CNS, they migrate and proliferate during and after injury and inflammation, phagocytose and process antigens, and once activated produce proinflammatory factors including the cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF) α. Pro-inflammatory mediators released from microglia not only are exocytotoxic, but also can secondarily activate astrocytes leading to a further induction of the expression of inflammatory factors.

In vitro and *in vivo* studies have shown that cannabinoids can act on glia and neurons to inhibit the release of pro-inflammatory cytokines and enhance the release of anti-inflammatory factors such as the cytokines IL-4 and IL-10. These observations suggest that these cannabinoids, as lipophilic molecules, can readily access the CNS and have the potential to ablate a variety of neuropathological processes that are associated with proinflammatory states.[22]

Multiple Sclerosis (MS)

Multiple Sclerosis is a chronic disease of the CNS, where autoimmunity is thought to drive the development of inflammatory lesions that induce the primary demyelination, which results in the inhibition of normal neurotransmission.[12]

This pathology causes muscle weakness, abnormal muscle spasms, change in sensation, difficulty in coordination and balance, cognitive impairment, problems with speech, swallowing and sight. The disease is characterized by degeneration of the myelin sheath that covers axons, in which T cells play a prominent role. The cells attack myelin with the resulting inflammatory process and so other immune cells are stimulated to produce soluble factors such as cytokines and antibodies.

The inflammatory process results in breakdown of the BBB leading to swelling, activation of macrophages and further production of cytokines and "cytotoxic" proteins such as metalloproteinases. Remyelination may occur in the early phase of the disease and it could be an opportunity for healing, but the oligodendrocytes that originally formed the myelin sheath cannot completely rebuild the destroyed myelin.[22]

CB1 and CB2 receptors expression is a specific markers of plaque cell subtypes in MS lesions. CB1 receptor is in fact expressed in cortical neurons,

oligodendrocytes, macrophages, and infiltrated T lymphocytes, whereas CB2 receptor is present in astrocytes and in microglial cells.

Several experimental models have shown that the activation of cannabinoid receptors in the inflammatory demyelinative process characterizing MS may cause a neuroprotective effect through a CB1 receptor-mediated inhibition of excitotoxicity and through a CB2 receptor-mediated inhibition of neuroinflammation.

The treatment with CB-mimetic substances suppresses inflammatory responses in the CNS of EAE (Experimental Autoimmune Encephalomyelitis) animals and improves their neurological symptoms by inhibiting the expression of proinflammatory cytokines[23], by reducing microglial activation, by abrogating major histocompatibility complex class II antigen expression, and by promoting remyelination **(Figure 17)**. Treatment with drugs able to modulate the endocannabinoid tone has in fact exerted efficacy. However, the use of Cannabis derivatives in MS patients should be considered cautiously, whereas the strategies to modulate endogenous CB system could have particular therapeutic interest.[24]

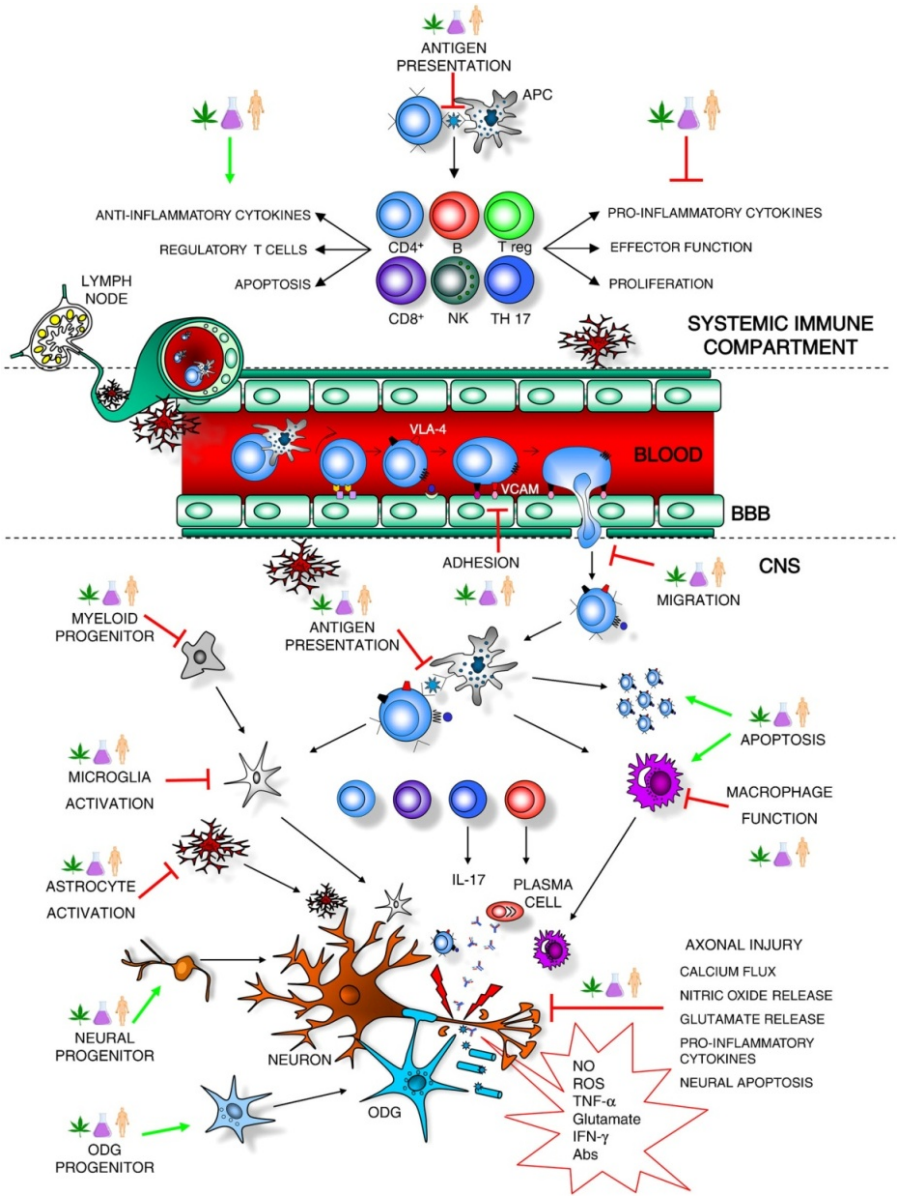


Figure 17. *The complex role of the cannabinoid system and possible therapeutic targets in MS-induced inflammation. Cannabinoids may combat inflammation by modulating cytokines, inflammatory mediators, T cell proliferation and effector function, B cell antigen presentation and activation of microglia and astrocytes. Moreover, they may limit the ability of leukocytes to cross the BBB and enter the brain by inhibition of molecules implicated in leukocyte endothelium trafficking.*[20]

Alzheimer's disease (AD)

AD is the most common chronic neurodegenerative disorder that causes senile dementia. The series of events that leads to AD is poorly understood and insights into its pathogenesis have been obtained primarily from assessment of human brain tissue at autopsy. Neurodegeneration in AD affects multiple neurotransmitter systems, including those of specific cholinergic, noradrenergic, serotonergeic, GABAergic, and glutamatergic neurons.

The defining neuropathologic features of the disease are the presence of extracellular neuritic amyloid plaques and intracellular neurofibrillary tangles (NFTs) in the brain. The appearance of these stigmata is accompanied by synaptic and neuronal loss and gliosis in the cerebral cortex and the limbic system. The amyloid plaques consist of extracellular aggregates of amyloid β (Aβ) peptides that often are surrounded by activated microglia and astrocytes. The NFTs consist of intraneuronal aggregates of paired-helical filaments that usually are composed of hyperphosphorylated microtubule-associated protein tau. As neurodegeneration progresses there is accelerated neurofibrillary tangle formation, neuroinflammation, and neuronal loss.

Alterations in components of the cannabinoid system have been observed in brains of Alzheimer's patients, suggesting that this system contributes to, or is altered by, the disease **(Figure 18)**. It was suggested that the losses in cannabinoid receptors, while related to generalized aging and/or the disease process, were not selectively associated with the pathology characteristic of AD. On the other hand, CB2 and FAAH have been reported to be selectively over-expressed in neuritic plaque-associated glia in AD brains.

Intracerebroventricular administration of cannabinoid receptor agonist WIN55212-2 prevented Aβ-induced activation of microglia, cognitive impairment, and loss of neuronal markers. In addition, treatment of microglia *in vitro* with CB receptor agonists HU-210, WIN55212-2 and JWH-133 resulted in blockage of their activation by Aβ peptide and abrogated neurotoxicity mediated by these cells. These results

are consistent with those that indicated that WIN-55212-2 exerted anti-inflammatory properties. It has been reported that stimulation of CB2 results in suppression of inflammatory mediators such as NO, cytokines, and chemokines that play a role in microglial cell-associated neuronal damage.[22,25]

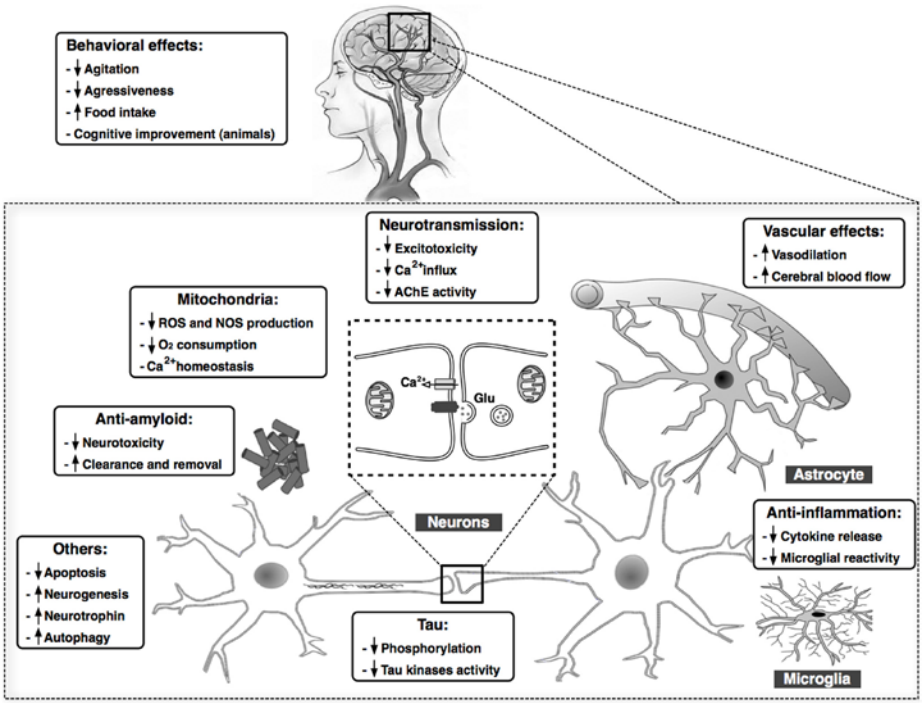


Figure 18. *Summary of the main findings demonstrating beneficial effects of cannabinoid compounds in AD models.*[25]

Amyotrophic Lateral Sclerosis (ALS)

ALS is a fatal neurodegenerative disease that primarily affects motor neurons in the spinal cord, brainstem, and motor cortex, leading to complete paralysis. It is characterized pathologically by progressive degeneration of cortical motor neurons (upper motor neurons) and clinically by muscle wasting, weakness, and spasticity.[22, 24]

This adult-onset disease occurs in both sporadic and familial forms. The familial ALS (FALS) form is inherited as an autosomal dominant trait and accounts for 5% to

10% of cases of ALS. FALS has been linked to mutations in the superoxide dismutase 1 (SOD1) gene that codes for a zinc and copper binding enzyme which neutralizes supercharged oxygen molecule (superoxide radical) byproducts of normal cellular metabolism.[22]

The pathogenesis of ALS is incompletely understood but the degeneration of motor neurons has been related to several mechanisms, including neurofilament accumulation, excitotoxicity, oxidative stress, and neuroinflammation with microglial activation. All these pathological events are potentially modulated by endocannabinoids.

It has been demonstrated that the levels of endocannabinoids are affected in experimental ALS. AEA and 2-AG are increased in the spinal cord of SOD1 transgenic mice and up-regulated with disease progression. This up-regulation might represent a neuroprotective response, not robust enough to counteract disease progression.

The ECS can interplay with the pathogenic events of ALS at different molecular and cellular levels. Neuroinflammation, microglial activation, oxidative stress, and excitotoxicity are involved in the pathophysiology of ALS and are potentially influenced by ECS. Disruption of extracellular glutamate homeostasis has been claimed to play a crucial role in ALS-associated excitotoxic damage, and activation of CB1 receptor protects against neuronal death induced by glutamate in other pathological conditions by reducing glutamate release from presynaptic terminals. Glutamate is known to induce the formation of reactive oxygen species, which inhibit glutamate uptake, thereby potentiating excitotoxic damage and oxidative stress in several pathological conditions including ALS. Microglial activation in the CNS is part of a neuroinflammatory reaction with deleterious effects on surrounding neurons. Endocannabinoids have been shown to reduce microglial activation as well as the expression and release of proinflammatory cytokines from microglia via CB2 receptor, providing a plausible explanation for their protective action in ALS.

CB2 receptor agonists or CB1 receptor antagonists should be identified as potential therapeutic agents in ALS. Neuronal CB1 receptors and microglial CB2 receptors might be suitable pharmacological targets in the early and late phases of ALS, respectively.[24]

Pain

Pain has a negative impact on the life quality in cancer patients. Almost half of all patients with cancer experience moderate to severe pain, and this increases in patients with metastatic or advanced stages of cancer. Cancer pain is often treated with oppioid drugs (codeine, morphine, and/or their synthetic analogues); however, these drugs have dose-limiting side-effects. The use of cannabinoids to treat cancer pain may provide a novel therapeutic approach, because they have the capability to inhibit pain transmission.

The manifestation of pain and its modulation is mediated by ascending and descending pathways. The descending pathways modulate neuronal activity in the ascending pathways, and can modulate effects on pain sensation. The midbrain periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) are the most studied regions and represent a significant system that contributes to pain modulation. The PAG projects to the RVM, which in turn sends its output to dorsal horn laminae, which are important in nociceptive function. The PAG/RVM system exerts bidirectional control over nociceptive processing and it is recognized as the central site of action of analgesic agents including cannabinoids.[26]

The components of the endocannabinoid system are found in regions involved in the transmission and modulation of nociceptive signaling[26]: CB1 receptors have shown particularly high levels in cells of the midbrain periaqueductal grey matter (PAG), and in the substantia gelatinosa of the spinal cord (receiving nociceptive

input from primary afferent neurons), which are key sites for modulating nociceptive information.[27]

Cannabinoid receptor agonists have long been known to exhibit antinociceptive activity in animal models of acute pain through the activation of CB1 receptors: they have greater potency in suppressing responses to noxious pressure or thermal stimuli.[26, 28] Recent experiments have demonstred that CB2 receptor-selective ligands are more active in animal models with hyperalgesia.

Health Canada approved Sativex in 2007, with conditions, as adjunctive analgesic treatment in adult patients with advanced cancer who experience moderate to severe pain during the highest tolerated dose of strong opioid therapy for persistent background pain.

Cancer

Cancer is a disease characterized by uncontrolled division of cells and their ability to spread. This unregulated growth is caused by damage to DNA, resulting in mutations, defects in cell cycle, and apoptotic machinery. Thus, agents that can modulate apoptosis to maintain steady-state cell population by affecting one or more signaling intermediates leading to induction of apoptosis can be useful for targeted therapy of cancer.

A significant advancement in cannabinoid use in cancer treatment came through the discovery of a potential utility of these compounds for targeting and killing tumors. In subsequent studies, molecular mechanisms for these effects were analyzed, and it was found that cannabinoids inhibited tumor cell growth and induced apoptosis by modulating different cell signaling pathways in gliomas[29] and lymphomas, prostate, breast, lung, skin, and pancreatic cancer cells.

The block of the G_1-S transition was ascribed to the inhibition of adenylyl cyclise and, consequently of cAMP-protein kinase A pathway and to the activation of

MAPK. Cannabinoids prevented the inhibition of RAF1 (caused by protein kinase A-induced Raf phosphorylation) and induced prolonged activation of the RAF1-MEK-ERK signaling cascade. Moreover, cannabinoids induce *de novo* synthesis of ceramides, a family of lipid molecules composed of sphingosine and a fatty acid, found in the cell membrane. Synthesis of ceramide occurs via activation of the enzyme ceramide synthase and leads to downstream activation of an extracellular regulated kinase (ERK) signaling cascade. This process results in cell cycle arrest and apoptosis. Activation of either CB1 or CB2 receptors triggers the ceramide-ERK signaling pathway to promote apoptosis. The increase in ceramide can also activate the p38 mitogen-activated protein kinase (p38MAPK) pathway, which can lead to apoptosis through multiple mechanisms. The over activation of ERK also promotes the induction of cyclin kinase inhibitor (p27/KIP1) which modulates regulatory molecules of the cell cycle (cyclins, cdks). Cell cycle arrest involves the up-regulation of the p53 protein which will differentially alter levels of pro- and anti-apoptotic proteins (i.e. increase the levels of the pro-apoptotic protein Bax and lower the levels of the anti-apoptotic protein Bcl2) which ultimately leads to activation of caspases that play an essential role in triggering apoptosis.

The diversified effects of cannabinoids in modulating cell signaling pathways are depicted in **Figure 19**.[30, 31]

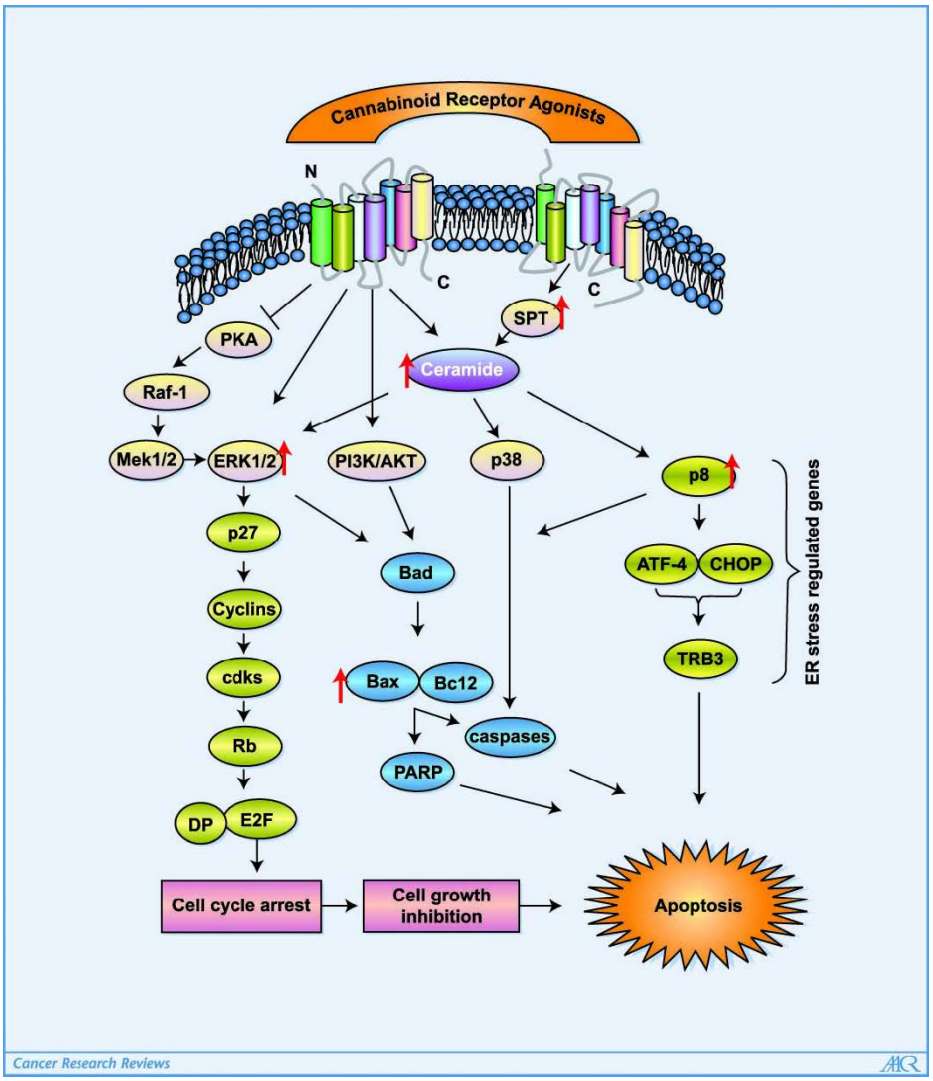


Figure 19. *Schematic representation of signaling pathways associated with cannabinoid receptor activation induced by its agonists.*[30]

Levels of endocannabinoids and their receptors are increased in cancer, a situation that frequently correlates with tumor aggressiveness. Accordingly, anandamide and 2-AG have been shown to be over-expressed in several types of tumors including glioblastoma multiforme, meningioma, pituitary adenoma, prostate and colon carcinoma and endometrial sarcoma.

Many different reports have shown that cannabinoid receptor agonists (derived from the plant, like THC, endogenous like 2-AG and anandamide or synthetic - with similar or different affinity for CB1 and CB2 receptors like WIN 55,2121-2 or JWH-

133) exert antitumor effects in experimental models of cancer supporting that pharmacological stimulation of CB receptors is antitumorigenic. Cannabinoid treatment promotes cancer cell death, impair tumor angiogenesis and block invasion and metastasis. The mechanism of cannabinoid anticancer action relies, at least largely, on the ability of these agents to stimulate autophagy-mediated apoptotic cancer cell death: autophagy is considered primarily a cytoprotective mechanism.

Cannabinoids have shown to normalize tumor vasculature. These effects seem to rely on the ability of cannabinoids to inhibit the stimulation of the vascular endothelial growth factor (VEGF) pathway. Thus, various components of the VEGF-activated pathway have been shown to be down-regulated in response to treatment with cannabinoids in different cancer types. Cannabinoid receptor activation inhibits migration and proliferation and induces apoptosis in vascular endothelial cells which contribute to the antiangiogenic effect of cannabinoids. In addition, cannabinoids have been shown to reduce the formation of distant tumor masses in animal models of spontaneous and induced metastasis.[30, 31, 32]

Cannabinoid receptor ligands

All the active compounds targeting the CB1 or CB2 receptor are classified into cannabinoids. The compounds could be classified as agonist, antagonist, and inverse agonists according to their functionality. The agonists produce an increase in the basal level of signaling after binding to the receptors, while the inverse agonists downregulate the signaling. In addition, the antagonists could prevent the agonists or inverse agonists modulating the receptors. Traditionally, cannabinoids can be divided into five classes based on the chemical scaffold, including classical cannabinoids, non-cassical cannabinoids, aminoalkylindoles, diarylpyrazoles, and endocannabinoids (or eicosanoids).[33]

Canabinoid receptor Agonists

1. *Classical Cannabinoids*

This group of cannabinoids consists of ABC-tricyclic dibenzopyran derivatives that are either compounds occurring naturally in the plant, *C. sativa*, or synthetic analogs of these compounds. The most investigated of the classical cannabinoids have been Δ^9-THC, Δ^8-THC, 11-hydroxyl-Δ^8-THC-dimethylheptyl (HU-210) and desacetyl-L-nantradol **(Figure 20)**. Of these, Δ^9-THC is the main psychotropic constituent of Cannabis. Δ^8-THC is also a psychotropic plant cannabinoid, whereas HU-210 and desacetyl-L-nantradol are synthetic cannabinoids. All these cannabinoids have been demonstrated to elicit cannabimimetic responses both *in vivo* and *in vitro*.

Δ^9-THC undergoes significant binding to cannabinoid receptors at submicromolar concentrations with similar affinities for CB1 and CB2 receptors. At CB1 receptor, it behaves as a partial agonist; at CB2 receptor is even less than its relative intrinsic activity. Δ^8-THC has affinities for CB1 and CB2 receptors that are similar to those of Δ^9-THC and behaves as a partial agonist at CB1 receptor. HU-210 is particularly

potent cannabinoid receptor agonist: its pharmacological effects *in vivo* are also exceptionally long lasting.[34, 35]

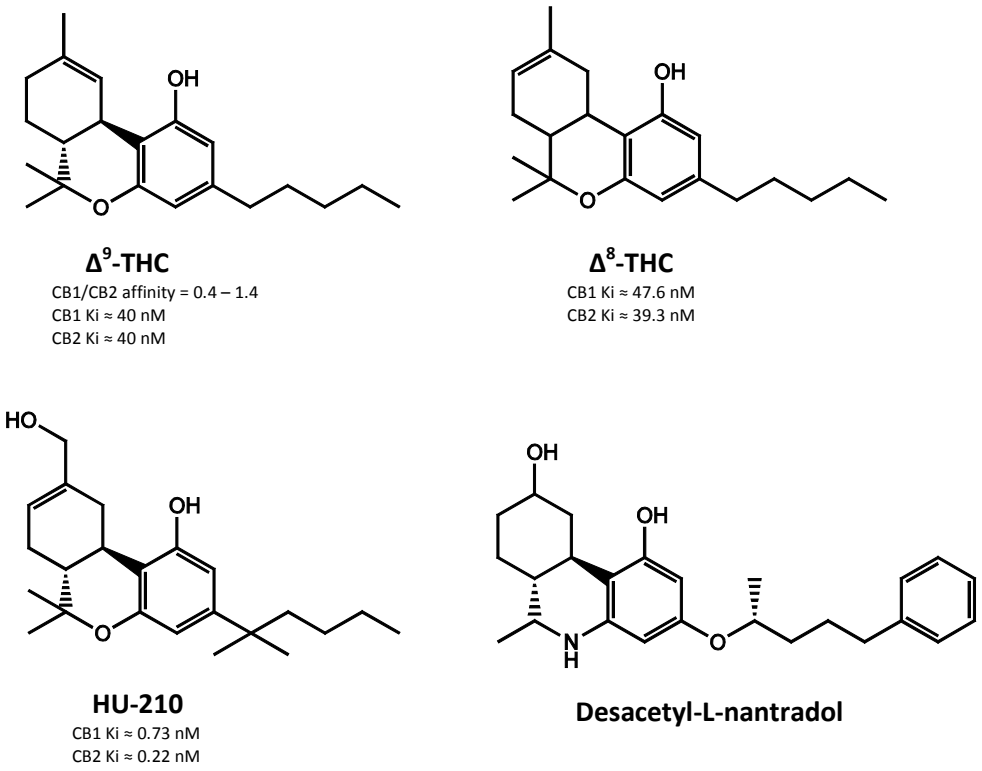


Figure 20. *Structures of synthetic classical cannabinoid receptor agonists.*

2. *Non-classical Cannabinoids*

Researchers at Pfizer synthesized new analogs lacking the dihydropyran ring of THC. CP47497 represents the prototypical compound of this series of AC-bicyclic and ACD-tricyclic cannabinoid analogs. Further developments have led to the bicyclic analog, CP55940 **(Figure 21)**, which has become one of the major cannabinoid agonists. It binds to CB1 and CB2 receptor with similar affinity and displays high activity *in vivo* being 10 to 50 times more potent than Δ^9-THC. CP55940 behaves as a full agonist for both receptor types.[34, 35]

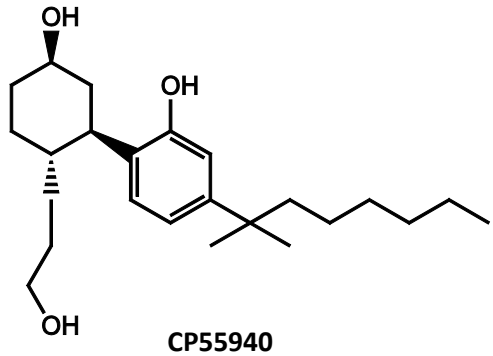
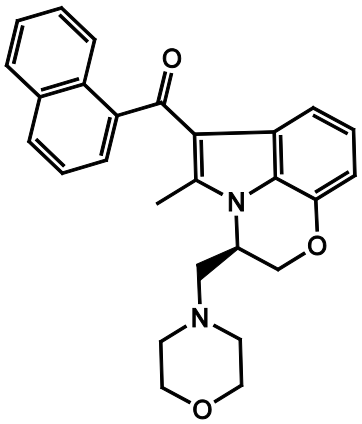


Figure 21. *Structure of non-classical cannabinoid receptor agonist CP55940.*

3. *Aminoalkylindoles*

Sterling Winthrop researchers reported a new family of aminoalkylindoles possessing cannabimimetic properties. This discovery resulted from the development of structurally constrained analogs of pravadoline, a series of compounds with reduced ability to behave as nonsteroidal anti-inflammatory agents that inhibit cyclooxygenase but increased ability to bind to the CB1 receptor. *R*-(+)-WIN55212 **(Figure 22)** is the most highly studied compound of the series. It displays high affinity for both cannabinoid receptors, with moderate selectivity in favour of the CB2 receptor, and exhibits high relative intrinsic activity at both CB1 and CB2 receptors. *In vivo*, it produces the full spectrum of pharmacological effects of THC and substitutes totally for other cannabinoids in discriminative stimulus tests, whereas its *S*-(-)-enantiomer, WIN55212-3, lacks activity both *in vivo* and *in vitro*.[34, 35]



***R*-(+)-WIN55212**
CB1/CB2 affinity = 6.8 - 30
CB1 Ki ≈ 1.9-123 nM

Figure 22. *Structure of aminoalkylindole cannabinoid receptor agonist R-(+)-WIN55212.*

4. *Endocannabinoids (Eicosanoids)*

The prototypic member of the eicosanoid group of cannabinoid receptor agonists is anandamide, which belongs to the 20:4, n-6 series of fatty acid amides. Anandamide behaves as a partial agonist at CB1 receptors and exhibits less relative intrinsic activity at CB2 than CB1 receptors.

2-arachidonylglycerol is an agonist for both CB1 and CB2 receptors and exhibits higher relative intrinsic activity than anandamide at both CB1 and CB2 receptors.

Structural modification of the anandamide molecule has led to the development of the first generation of CB1-selective agonists. Notable examples are R-(+)-methanandamide, arachidonyl-2'-chloroethylamide (ACEA)[35], arachidonylcyclopropylamide (ACPA)[35], and O-1812 **(Figure 23)**.[34]

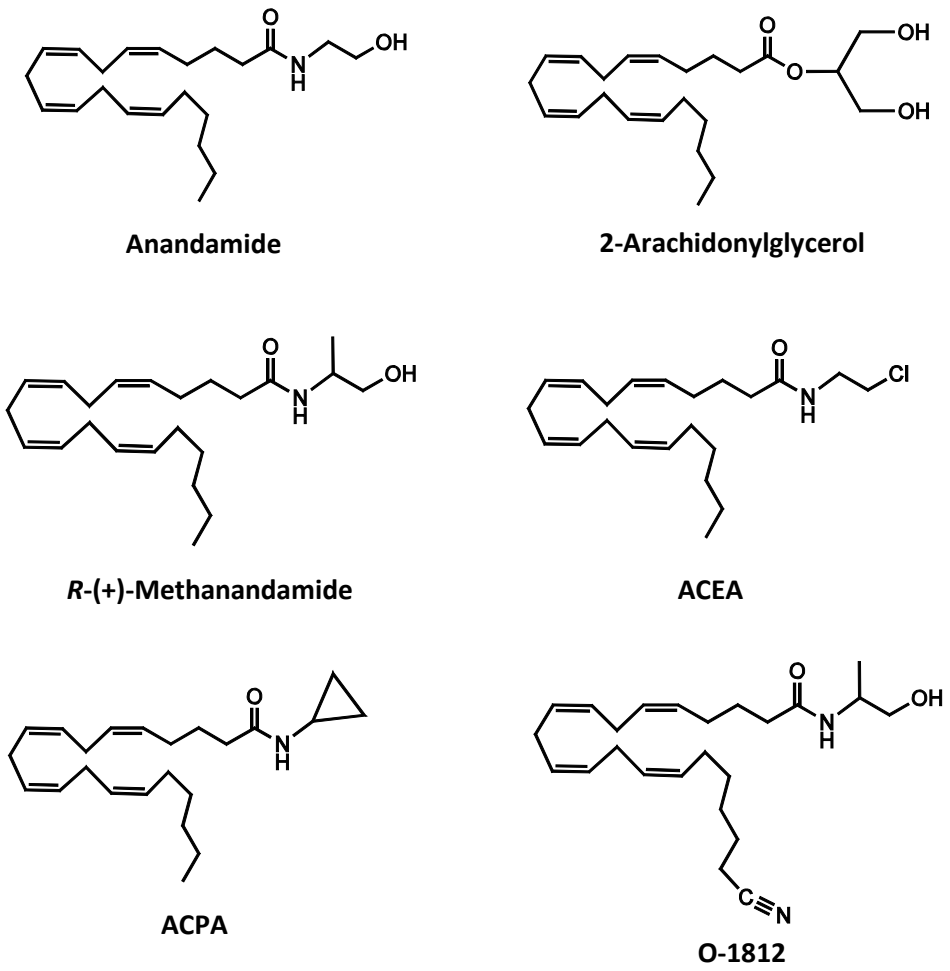


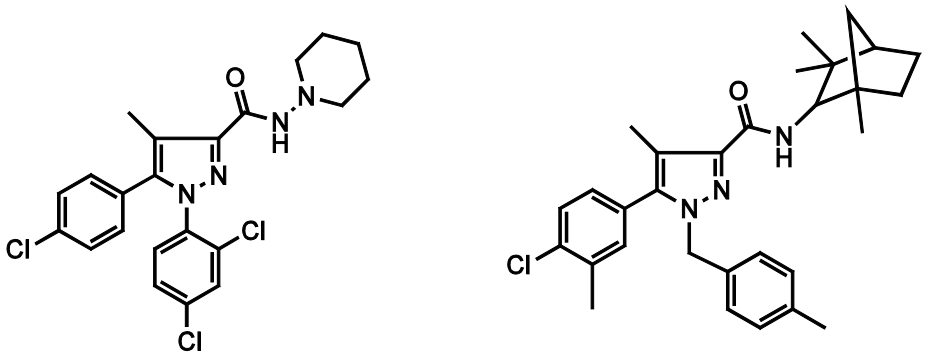
Figure 23. *Structures of endocannabinoids and synthetic cannabinoid receptor agonists.*

Canabinoid receptor Antagonists/Inverse Agonists

1. *Diarylpyrazoles*

The prototypic members of this series of compounds are the Sanofi compounds SR141716A (Rimonabant, Acomplia®), a potent CB1-selective ligand, and SR144528, a potent CB2-selective ligand **(Figure 24)**. These ligands readily prevent or reverse effects mediated respectively by CB1 and CB2 receptor agonists. Although these effects of the arylpyrazole antagonists may be attributable to the inhibition of endogenously produced agonists in the biological preparation, there is evidence that SR141716A and SR144528 can evoke inverse agonist responses. This

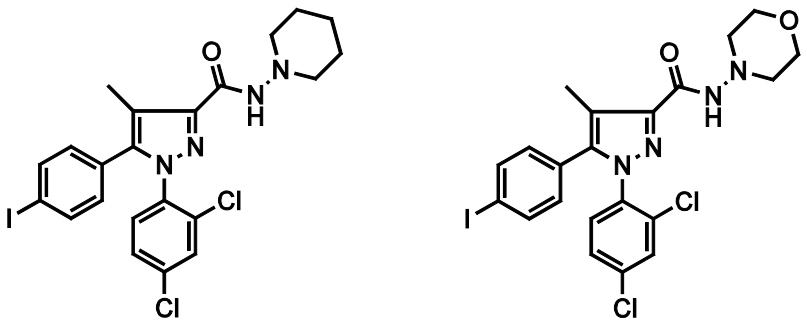
notion rests on the ability of the CB1 and CB2 receptor to exhibit signal transduction activity in the absence of endogenous or exogenous agonists (constitutive activity). As such, arylpyrazoles can behave as *"inverse agonists"* to reduce the constitutive activity of these signal transduction pathways. In some experiments, SR141716A has been found to be more potent in blocking the actions of CB1 receptor agonists than in eliciting inverse cannabimimetic responses by itself.[34, 35, 36]



SR-141716A **SR-144528**

Figure 24. *Structures of cannabinoid receptor antagonists/inverse agonists.*

Two analogs of SR141716A that have also been used to block CB1 receptor-mediated effects: AM251[37] and AM281[37] **(Figure 25)**. AM281 has 350 times greater affinity for CB1 than CB2 receptor, and both analogs share the ability of SR141716A to attenuate responses to established cannabinoid receptor agonists.[34, 35]



AM-251 **AM-281**

Figure 25. *Structures of cannabinoid receptor antagonists/inverse agonists.*

As a therapeutic target, the endocannabinoid system has found to date modest success. However, a number of new findings, coupled with current efforts for cannabinoid-based drug development, point to a more successful future. The ubiquitous presence of the endocannabinoid system presents difficulties in targeting it for therapeutic gain. For this reason, it is important that indications for cannabinergic drug development be explored very thoughtfully. In this regard, a better understanding of cannabinoid receptor-related functional selectivity should assist in the development of drugs with safer pharmacological profiles and also identify additional therapeutic opportunities.[37]

Aim of the Thesis

The endocannabinoid system is now known to be an ubiquitous neuromodulatory system with wide-ranging actions. It consists of endogenous cannabinoids (endocannabinoids), cannabinoid receptors and synthetic and degrading enzymes responsible for synthesis and degradation of endocannabinoids.

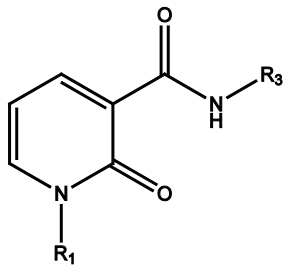
Endocannabinoids (EC) are lipid messengers derivatives of integral components of the cellular membranes: anandamide and 2-arachidonylglycerol are the best characterized. Their levels are maintained mainly by two catabolic enzymes: the fatty acid amide hydrolase (FAAH) and the monoacylglycerol lipase (MAGL).

Cannabinoid receptors 1 and 2 (CB1 and CB2) belong to the superfamily of G-protein-coupled receptors. CB1 receptor is predominant in the central nervous system while CB2 receptor is located mainly in immune cells, such as macrophages, microglia, and B and T cells. As CB1 receptor mediates most, if not all, of the psychoactive effects of cannabinoids, CB2 receptor selective ligands are attractive as therapeutics because they would presumably lack this psychoactivity. Therefore, therapeutic strategies might include the use of cannabinoid receptor agonists and/or antagonists, but also the blockage of hydrolytic enzymes degrading ECs, the inhibition of EC transporters (EMT) and the inhibition of EC uptake. However, the current comprehension of the complexity of the endocannabinoid system seems to indicate a need of multitarget drugs, which exert their pro-cannabinoid activities by means of more than one mechanism of

action. In fact, these molecules could offer the advantage of modulating the ECS in a safer and more therapeutically efficacious way.

The growing interest in the endocannabinoid system has led several research groups to design and develop new selective ligands that could have a target in the ECS, avoiding the psychoactive effects.

In a previous research program aimed at obtaining CB2 receptor selective ligands, a series of 2-oxo-1,2-dihydropyridine-3-carboxamide derivatives with general structures **A** (**Figure 26**) were developed.[39, 40] These compounds derived from a structural simplification of a class of 1,8-naphthyridine-3-carboxamides previously designed in laboratory where I performed my thesis, as a series of selective CB2 receptor agonists, which exhibited interesting pharmacological properties like anti-proliferative activity in cancer cells and immuno-modulatory activity in models of neuroinflammation.[40]



A

Figure 26. *2-Oxo-1,2-dihydropyridine-3-carboxamide derivatives **A***.

Compounds **A** presented all the features required for the preservation of good CB_1/CB_2 selectivity: a central lipophilic core (the 2-oxo-1,2-dihydropyridine nucleus) connected with a nonaromatic carboxamide group in position 3, and a lipophilic substituent at the 1-position with an H-bond acceptor atom.

2-Oxo-1,2-dihydropyridine-3-carboxamide derivatives **A** exhibited high affinity and interesting selectivity towards the CB2 receptor. Regarding to the structural modifications in the position 1 of the 2-oxo-pyridine nucleus, the *p*-fluorobenzyl

substituent results important in determining the maintenance of the affinity towards both receptor subtypes: in fact derivatives with this substituent in N1-position showed the highest CB2 receptor affinity.

Replacement of the cycloheptylamide with other cycloamide groups in position 3 of the 2-oxo-pyridine nucleus of compound **A** decreased the receptor affinities.

Moreover, the shift of the substituent in N-1 position to the oxygen in position 2 of the heterocyclic nucleus gave derivatives that showed low affinity.[40]

The significant affinity towards CB2 receptor displayed by some of derivatives with general structure **A** made this new scaffold useful as initial building block for designing CB2 receptor ligands. Furthermore some of these CB2 receptor ligands were effective on different tumor cell lines (human breast carcinoma, human prostate carcinoma, human gastric adenocarcinoma, glioblastoma). Antiproliferative effect of these compounds was mediated by the CB2 receptor, indeed they decreased the CB2 receptor expression levels and their effect was reverted by the CB2 antagonist.

Subsequently, the introduction of a substituent in position 5 of the heterocyclic nucleus (compounds **B**, **Figure 27**) showed an interesting effect on the activity on the CB2 receptor. Indeed, the nature of this substituent determined the control of the switch among the different types of pharmacological modulation: agonism, inverse agonism or antagonism on the receptor. In particular, replacing the hydrogen atom in position 5 with a phenyl group (**B1**) or with a 4-methoxyphenyl group (**B2**), the CB2 receptor activity shifted from agonism to inverse agonism and antagonism, respectively. Conversely, substituting the hydrogen with a bromine atom (**B3**) the CB2 receptor activity did not vary.

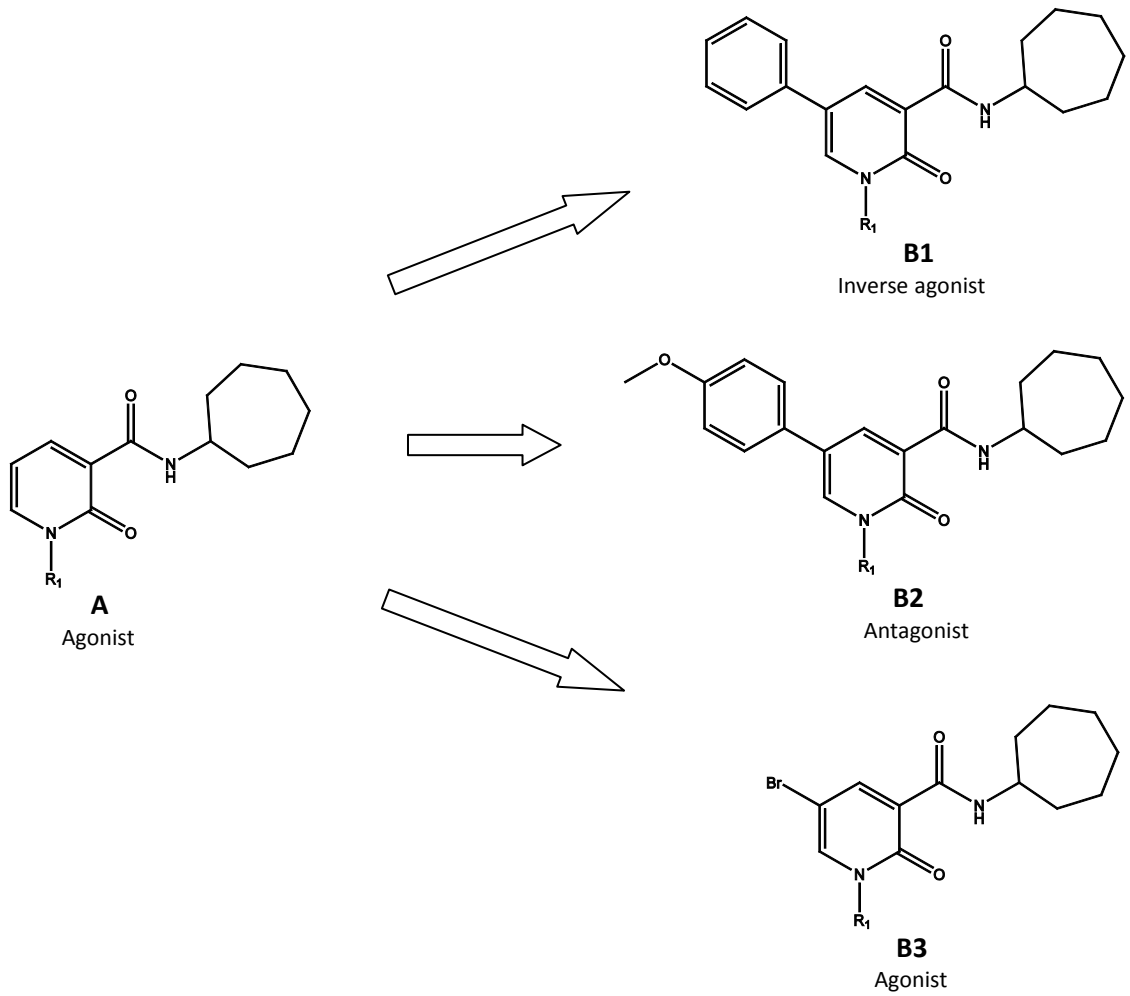


Figure 27. *The position C-5 of the 2-oxopyridine nucleus permitted the identification of the "key" position to be substituted on the 1,2-dihydro-2-oxopyridine scaffold responsible for functionality switch within this series of compounds.*

Subsequently the 5-substituted 2-oxo-1,2-dihydropyridine-3-carboxamide derivatives **B** were modified through the insertion of a methyl group at the position C-6 or C-4 to obtain compounds **C** and **D** (**Figure 28**).

These compounds were tested in order to evaluate the influence of a small substituent at the position 4 or 6 of the heterocyclic nucleus on the CB receptors affinity.

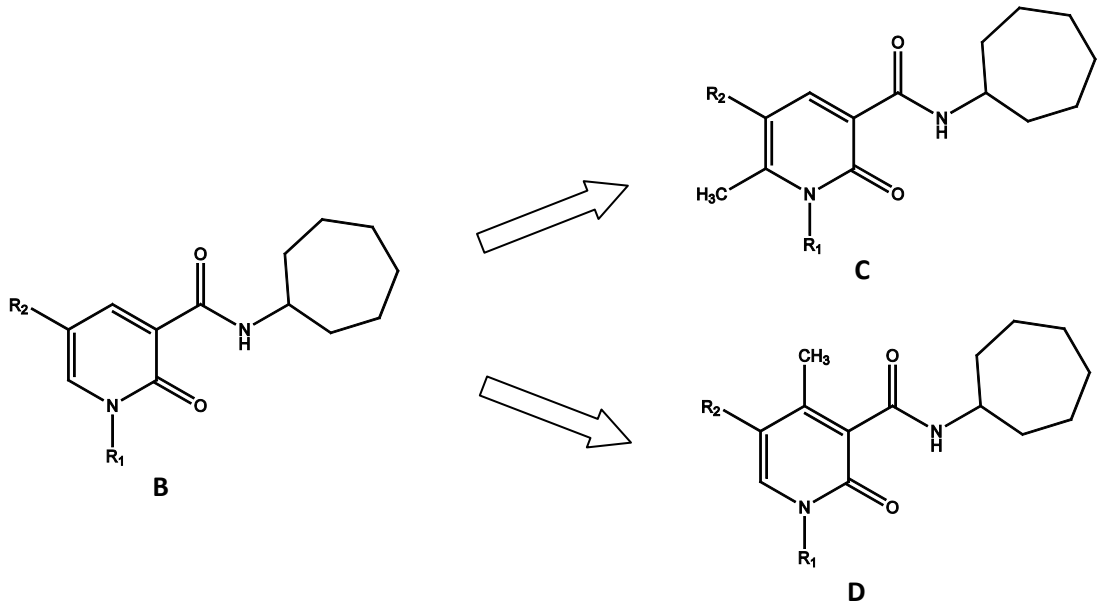
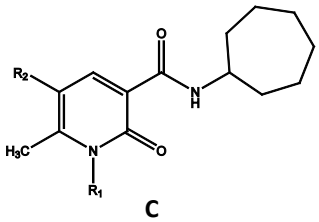


Figure 28. *Development of compounds with general structures **C** and **D**.*

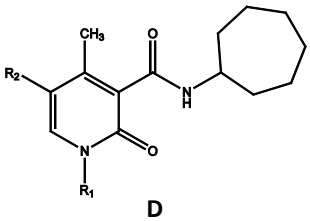
Compounds of both series showed not only the best binding properties at CB2 receptor (**Table 3**), but also the same behaviour towards these receptors in term of functional activity.

Furthermore, the 6-methyl-2-oxo-1,2-dihydropyridine-3-carboxamide derivatives **C** showed also inhibition of AEA and 2-AG uptake with IC_{50} values in the nM range. Finally some of these ligands showed to inhibit the fatty acid amide hydrolase (FAAH) (**Table 4**) and none of the tested compounds induced inhibition of MAGL and ABHDs activities.

Table 3 Radioligand binding data of 6-substituted-2-oxo-1,2-dihydropyridine derivatives



C



D

	R_1	R_2	K_i (nM)		
			CB_1 [a]	CB_2 [b]	SI [c]
C1	*p*-Fluorobenzyl	H	14.2	9.4	3.3
C2	*p*-Fluorobenzyl	Phenyl	176	5.6	31.4
C3	*p*-Fluorobenzyl	*p*-Methoxyphenyl	9.8	26.2	0.4
C4	*p*-Fluorobenzyl	Br	8.9	2.8	3.1
C5	*p*-Fluorobenzyl	F	5.2	5.4	0.9
C6	*p*-Fluorobenzyl	Cl	4	0.6	6.8
C7	*p*-Fluorobenzyl	I	1921	1230	1.6
D1	*p*-Fluorobenzyl	H	9.2	0.4	23
D2	*p*-Fluorobenzyl	Br	4.5	1.1	4.1
D3	*p*-Fluorobenzyl	Phenyl	23.1	3.9	5.8
D4	*p*-Fluorobenzyl	*p*-Methoxyphenyl	3.2	7.36	0.4

[a] Affinity of compounds for CB1 receptor was evaluated using membranes from CHO cells transfected and [^{3}H]CP-55,940.

[b] Affinity of compounds for CB2 receptor was evaluated using membranes from CHO cells transfected and [^{3}H]CP-55,940.

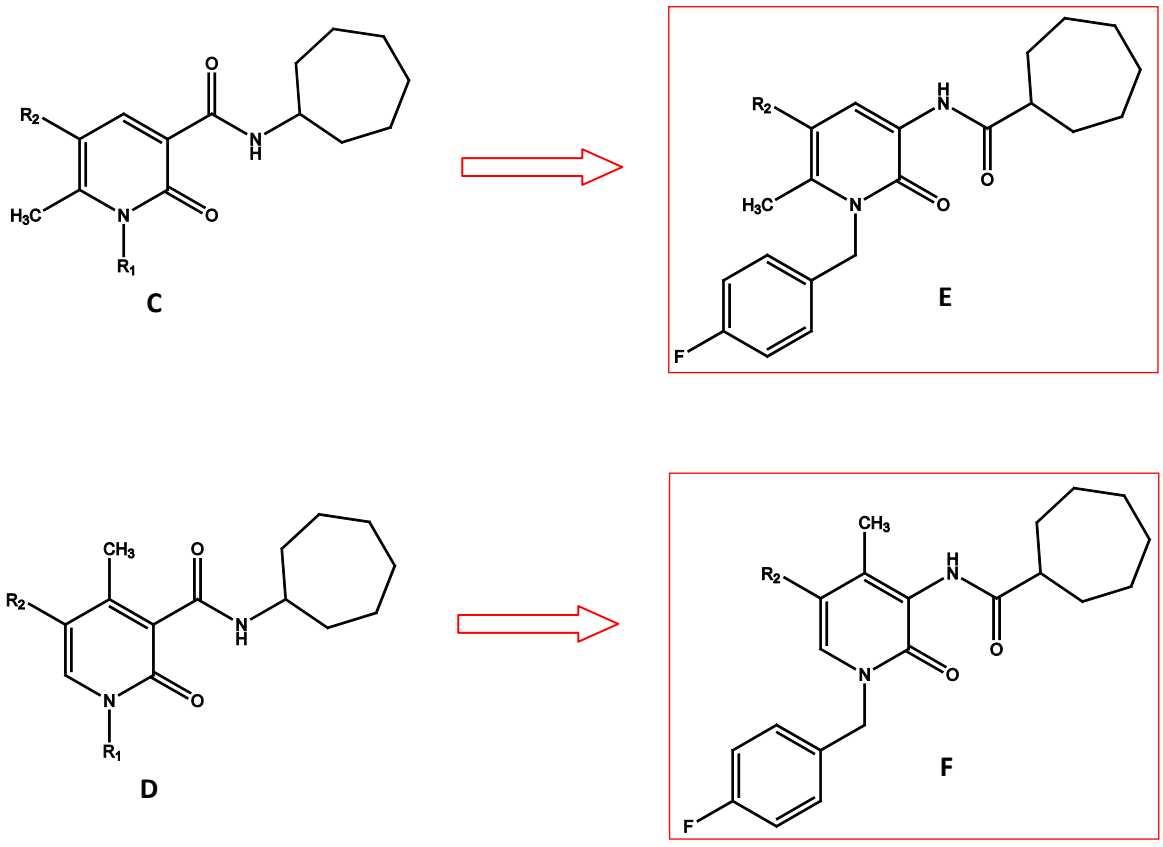
[c] Selectivity index for CB2 receptor calculated as K_i(CB1R)/K_i(CB2R) ratio.

Table 4 Evaluation of 6-methyl-2-oxo-1,2-dihydropyridine derivatives on FAAH and on putative endocannabinoid transporter (EMT)

	R_1	R_2	EC_{50} (μM)	EC_{50} (μM)	Selectivity over FAAH
			EMT	**FAAH**	
C1	*p*-Fluorobenzyl	H	≥ 10	4.37	-
C2	*p*-Fluorobenzyl	Phenyl	0.098	0.086	0.9
C3	*p*-Fluorobenzyl	*p*-Methoxyphenyl	0.082	0.246	3
C4	*p*-Fluorobenzyl	Br	1.63	0.855	0.5
D1	*p*-Fluorobenzyl	Phenyl	-	213	

Therefore some of these compounds, which are able to modulate more than one target of the ECS, might be exploited to investigate the multi-target approach for modulating the ECS and then they could be useful for the treatment of different pathological conditions like cancer and neurodegenerative diseases.

In the light of these good results, with the aim to deepen the structure activity relationship (SAR) of this series of compounds, during my thesis work, derivatives with general structures **E** and **F** were designed and synthesized as analogs of compounds **C** and **D** in which the amide group in position C-3 was substituted by the corresponding reverse amide (**Figure 29**).

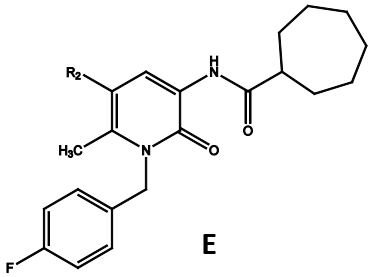


R_2 = H, Br, Cl, F, phenyl, *p*-methoxyphenyl.

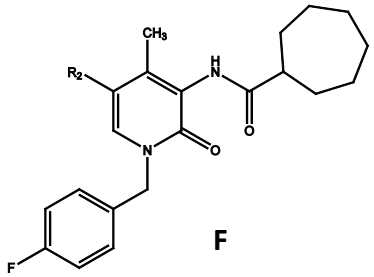
Figure 29. *Development of compounds with general structures **E** and **F**.*

Thus, I designed and synthesized compounds **E1-E6** and **F1-F5** (**Table 5**).

Table 5 Compounds designed and synthesized during my thesis work



E



F

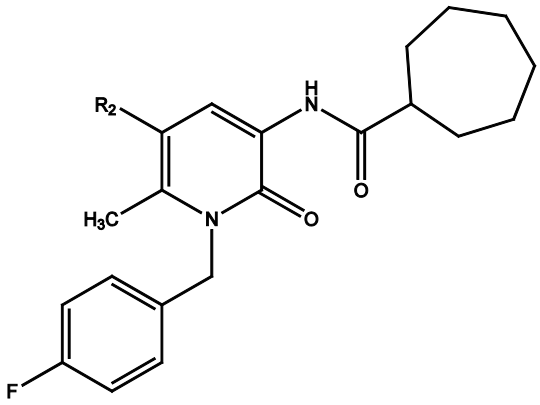
E		**F**	
	R_2		**R_2**
E1	Phenyl	**F1**	H
E2	H	**F2**	Br
E3	Br	**F3**	*p*-Methoxyphenyl
E4	*p*-Methoxyphenyl	**F4**	Phenyl
E5	Cl	**F5**	Cl
E6	F		

All the substituents of compounds **E** and **F** have been chosen considering the best results obtained with previous compounds. The novel ligands are characterized by a *p*-fluorobenzyl group in position 1 of the pyridine nucleus and a cycloheptanecarboxamide in position 3. Hydrogen, bromine, chloro, fluoro, phenyl and *p*-methoxyphenyl are chosen as substituents in position 5 (R_2) of the pyridine nucleus.

Synthesis

Synthesis of N-(6-methyl-2-oxo-1,2-dihydropyridin-3-yl)cycloheptanecarboxamide derivatives E

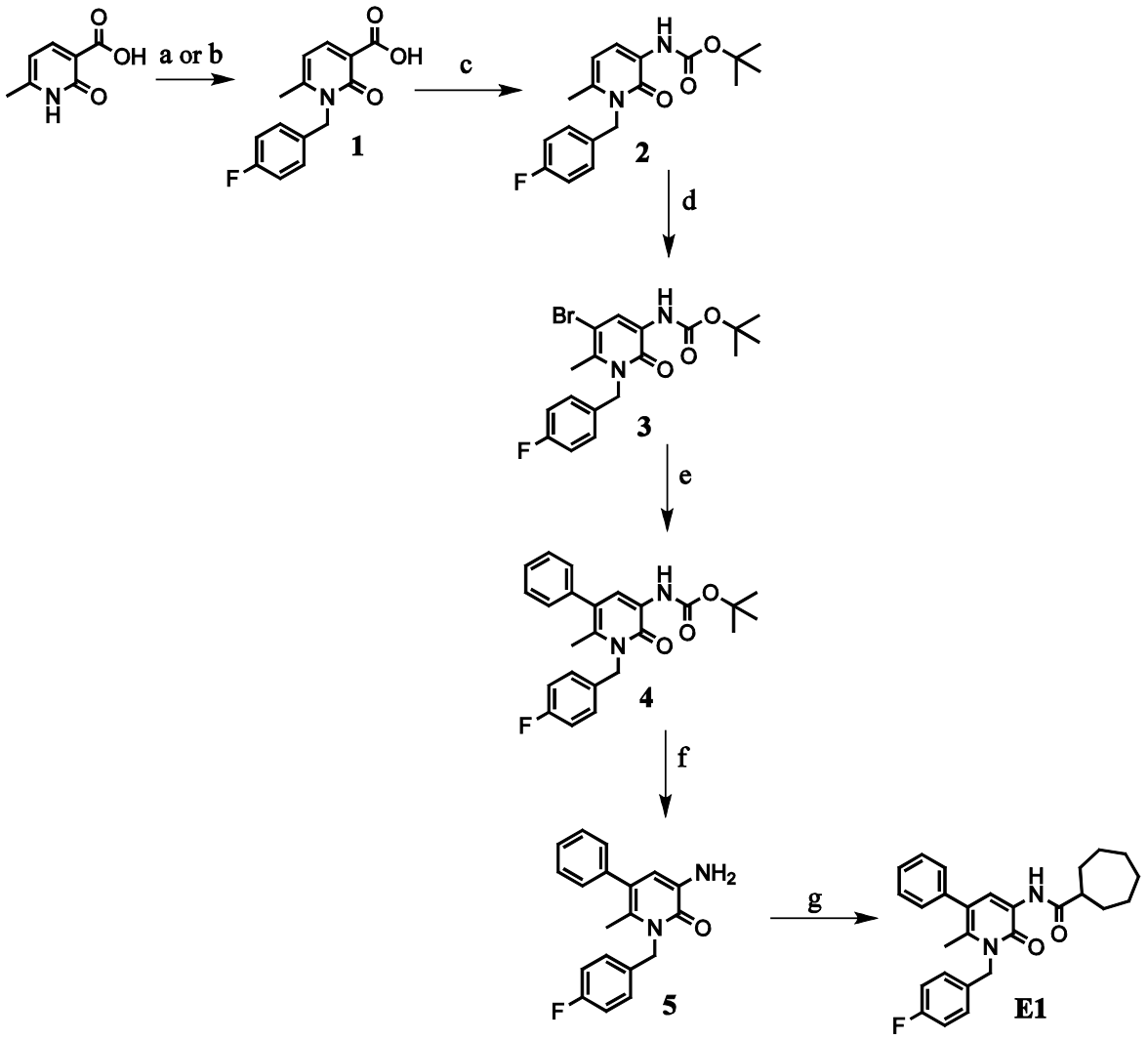
The synthesis of *N*-(6-methyl-2-oxo-1,2-dihydropyridin-3-yl)cycloheptanecarboxamide derivatives **E1-E6** (**Figure 30**) was initially planned following the synthetic pathway showed in **Scheme 1**. This synthetic route was used only to synthesize compound **E1**, as the yields were very low.



E1 R_2 = Phenyl;
E2 R_2 = H;
E3 R_2 = Br;
E4 R_2 = *p*-Methoxyphenyl;
E5 R_2 = Cl;
E6 R_2 = F.

Figure 30. *Design of compounds **E1-E6**.*

Scheme 1. Synthetic route for compounds **E**



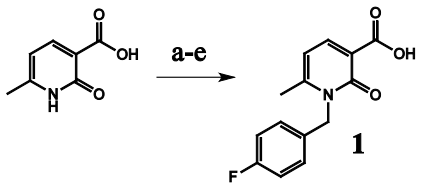
Reagents and conditions: **a) 1)** NaH 60%, DMF, 4-fluorobenzyl chloride, 50°C, 24h **2)** NaOH 10% reflux, 4h **3)** HCl 10%; **b) 1)** NaH 60%, LiBr, 4-fluorobenzyl chloride, DMF, DME, 65°C, overnight **2)** NaOH 60% reflux, 4h **3)** HCl; **c)** *tert*-butyl alcohol, diphenylphosphoryl azide, potassium *tert*-butoxide, reflux, overnight; **d)** Br_2, $CHCl_3$, rt, overnight; **e)** Triphenylphosphine, $Pd(OAc)_2$, toluene, K_2CO_3, phenylboronic acid, 100°C, overnight; **f)** HCl 37%, rt, 1h; **g) 1)** Cycloheptancarboxilic acid, $SOCl_2$, 76°C, 3h **2)** Toluene, NEt_3, 0-25°C, overnight.

The synthetic route started with a *N*-alkylation reaction carried out treating a solution of commercially available 6-methyl-2-oxo-1,2-dihydropyridine-3-carboxylic acid in anhydrous DMF with NaH 60%, and, after 2 hours, adding 4-

fluorobenzyl chloride. The reaction mixture was stirred at 50°C for 24 hours. The treatment of the crude residue with NaOH 10% and then with HCl 10% gave the desired *N*-alkylated derivative **1** in very low yields. To increase the yield, the same reaction was performed increasing the temperature and/or the percentage of NaH 60%, but unsuccessfully.

Furthermore the reaction conditions were changed: LiBr was used in addiction to NaH 60%. These conditions favour the synthesis of *N*-functionalized product.[42, 43] In these conditions yield of compound **1** increased (**Table 6**).

Table 6 Attempts, conditions and yields of *N*-alkylation



Attempts	Reagents and conditions	Yields
a	1) DMF, NaH 60% (1.2 eq), rt, 2h 2) 4-fluorobenzyl chloride, 50°C, 24h	8.5%
b	1) DMF, NaH 60% (1.2 eq), rt, 2h 2) 4-fluorobenzyl chloride, 70°C, 24h	17%
c	1) DMF, NaH 60% (2 eq), rt, 2h 2) 4-fluorobenzyl chloride, 70°C, 24h	5%
d	1) DMF, NaH 60% (2.5 eq), rt, 2h 2) 4-fluorobenzyl chloride, 70°C, 24h	9 – 17%
e	1) DMF/DME in ratio 1:4, NaH 60%, LiBr (2 eq), 0°C-rt 2) 4-fluorobenzyl chloride, 65°C, overnight	30 – 40%

Compound **1** was submitted to Curtius reaction (**Figure 31**), which was performed using *tert*-butyl alcohol, diphenylphosphoryl azide, potassium *tert*-butoxide and refluxing the mixture. The purification of the crude residue by flash chromatography provided the derivative **2** in 20% yield.

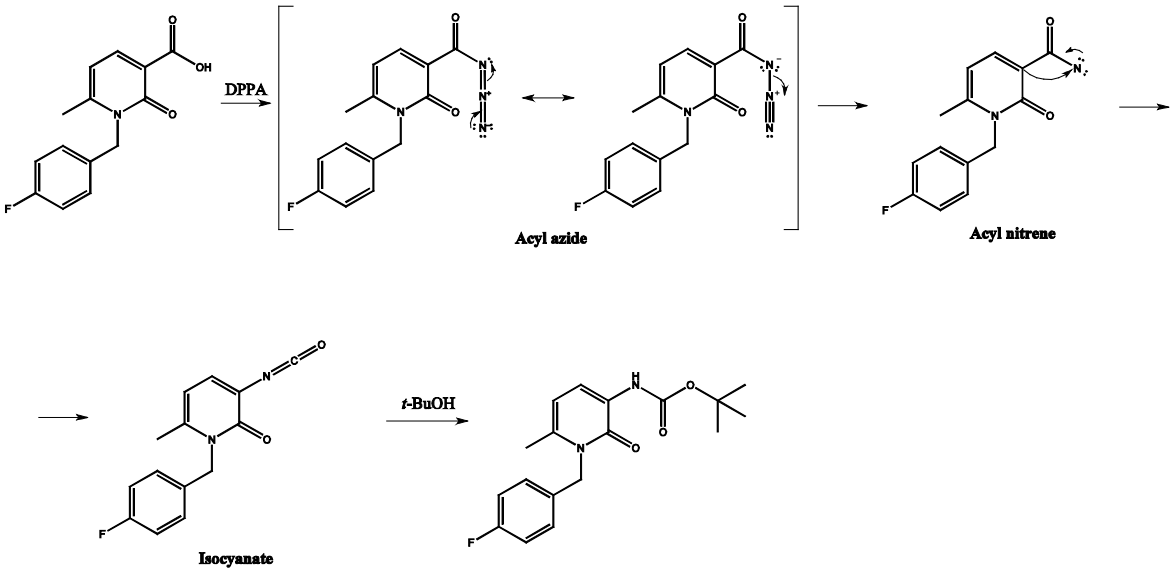
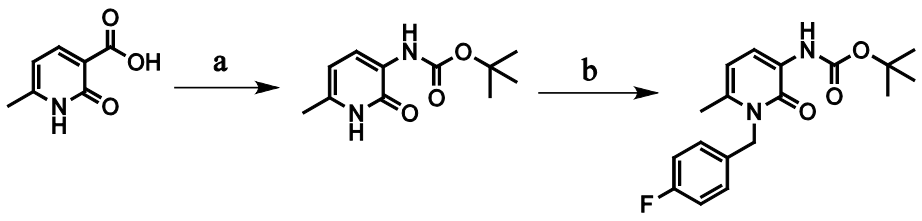


Figure 31. *Mechanism of Curtius reaction.*

In order to improve yields, Curtius reaction was carried out before *N*-alkylation (**Scheme 2**) but in this way the yields did not change. Furthermore each steps of this procedure required a purification by flash chromatography, while in the first case showed in **Scheme 1** only a purification by flash chromatography was needed.

Scheme 2. Attempt to improve yields reversing the first two steps of **Scheme 1**



Reagents and conditions: a) 1) DPPA, NEt_3, dioxane, 100°C, 2h **2)** *tert*-butyl alcohol, reflux, overnight; **b)** NaH 60%, DMF, 4-fluorobenzyl chloride, 70°C, overnight.

Compound **2** was treated with a solution of bromine in $CHCl_3$ (**Scheme 1**) to afford the corresponding derivative **3**.[44]

The 5-bromo derivative **3** was then subjected to a Suzuki-Miyaura cross-coupling reaction[45] (**Figure 32**) with the phenylboronic acid in toluene in the presence of tetrakis(trisphenylphosphine)palladium(0) as catalyst and anhydrous potassium carbonate as base, stirred at 100°C overnight to afford compound **4**.[46] Palladium(0) compound required in this cycle, $Pd(PPh_3)_4$, was prepared *in situ* using triphenylphosphine (PPh_3) and palladium acetate ($Pd(OAc)_2$). Compound **4** was purified by flash chromatography (**Scheme 1**).

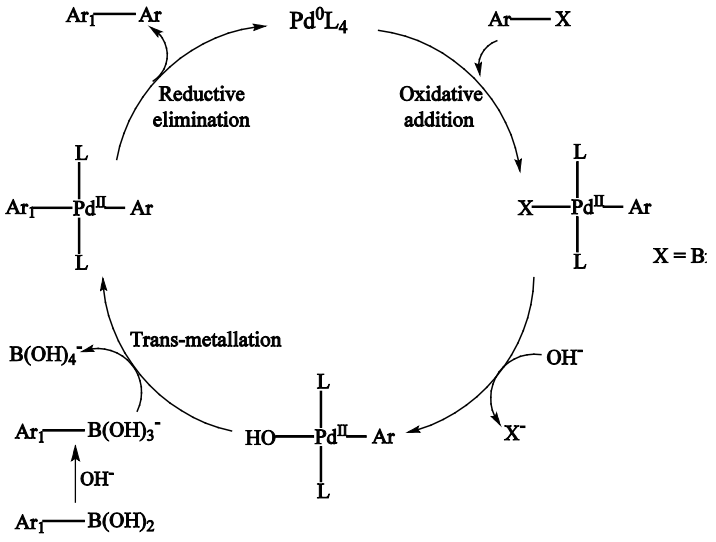
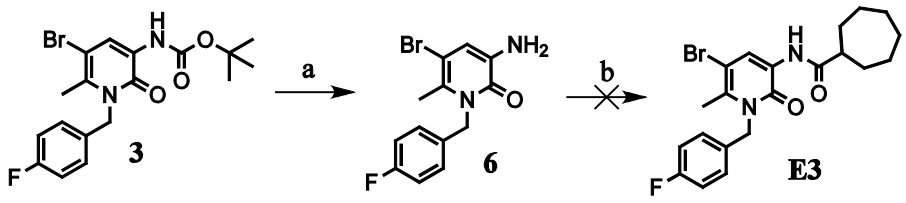


Figure 32. *Suzuki-Miyaura cross-coupling reaction mechanism.*[45]

As described in **Scheme 1**, compound **E1** was obtained *via* a two-step procedure starting from compound **4**. First of all, a deprotection of the amine group through a hydrolysis with HCl 37% occurred and compound **5** was obtained. The second step involved the formation of the amide moiety in position 3 through the reaction between compound **5** and the suitable acyl chloride in toluene and triethylamine at 0°C. The acyl chloride was obtained by the reaction between cycloheptanecarboxylic acid and $SOCl_2$ at 76°C for 3 hours.
The mixture was purified by flash chromatography to afford compound **E1** (**Scheme 1**).

As reported in **Scheme 3**, compound **3** was converted in compound **6** by a hydrolysis carried out with HCl 37%.
Unfortunately the subsequent reaction of amide formation through the acyl chloride to obtain compound **E3**, failed.

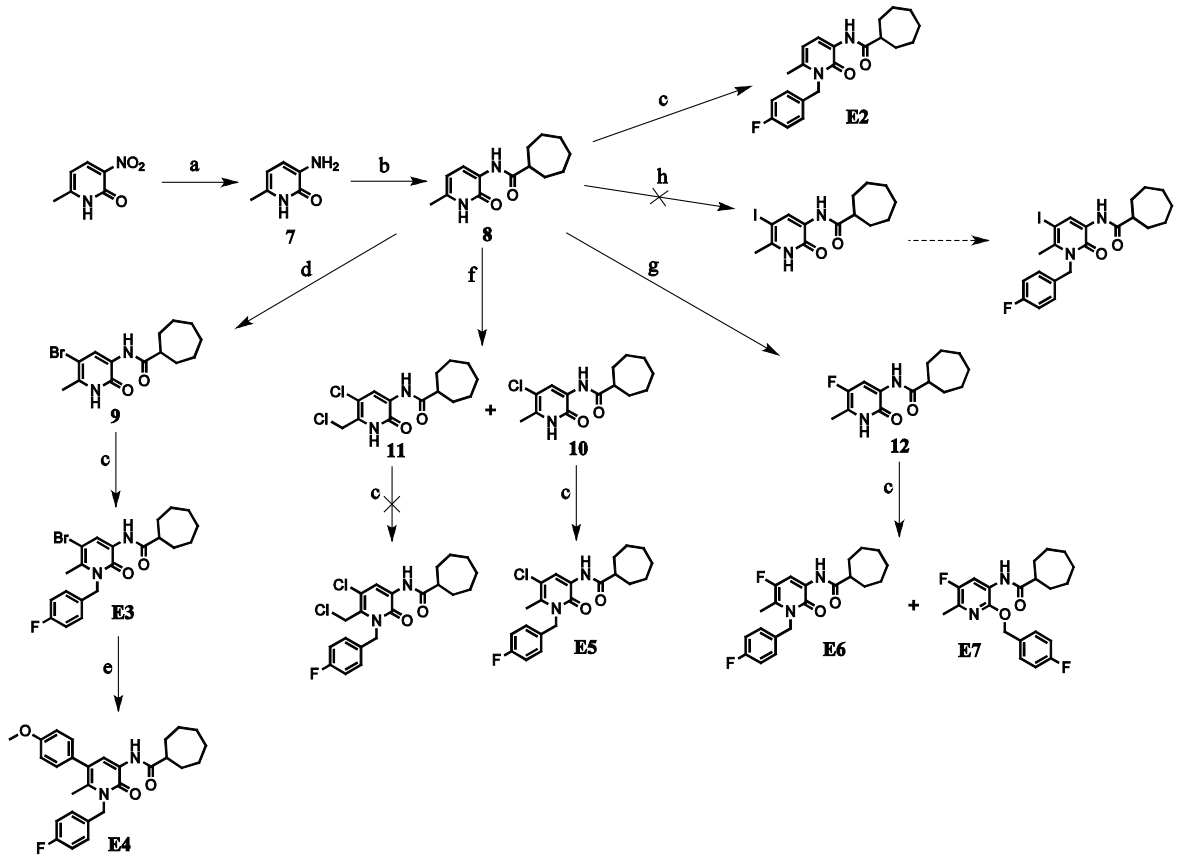
Scheme 3. Attempt for preparation of compound **E3**



Reagents and conditions: a) HCl 37%, rt, 1h; **b) 1)** Cycloheptancarboxilic acid, $SOCl_2$, 76°C, 3h **2)** Toluene, NEt_3, 0-25°C, overnight.

Alternatively, a new synthetic pathway was developed to afford all the other desired compounds **E** (**Scheme 4**).

Scheme 4. Synthetic route for compounds **E2-E7**



Reagents and conditions: **a)** H_2, Pd/(C), MeOH, rt, overnight; **b)** Cycloheptancarboxilic acid, TBTU, NEt_3, DMF, 0°C-rt, overnight; **c)** NaH 60%, LiBr, 4-fluorobenzyl chloride, DMF, DME, 65°C, overnight; **d)** Br_2, $CHCl_3$, rt, overnight; **e)** Triphenylphosphine, $Pd(OAc)_2$, toluene, K_2CO_3, *p*-methoxyphenylboronic acid, 110°C, overnight; **f)** *N*-chlorosuccinimide, ACN, reflux, overnight; **g)** Selectfluor™, ACN, reflux, overnight; **h)** *N*-iodosuccinimide, ACN, reflux, overnight.

The key intermediate of this synthetic route is *N*-(6-methyl-2-oxo-1,2-dihydripyridin-3-yl)cycloheptanecarboxamide **8**, useful for the synthesis of all the other desired compounds **E2-E7**.

This derivative was obtained *via* a two-step procedure. Firstly, the commercially available 2-hydroxy-6-methyl-3-nitropyridine was undergone to catalitic hydrogenation, using Pd/C as a catalyst, to afford compound **7**.

After, cycloheptanecarboxylic acid was treated with the coupling agent 2-[(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate] (TBTU) at 0 °C, in presence of triethylamine to afford the corresponding activated benzotriazolyl-ester (**Figure 33**), and then 2-hydroxy-6-methyl-3-aminopyridine (**7**) was added to give the desired carboxamide derivative **8**, which was purified by flash chromatography.

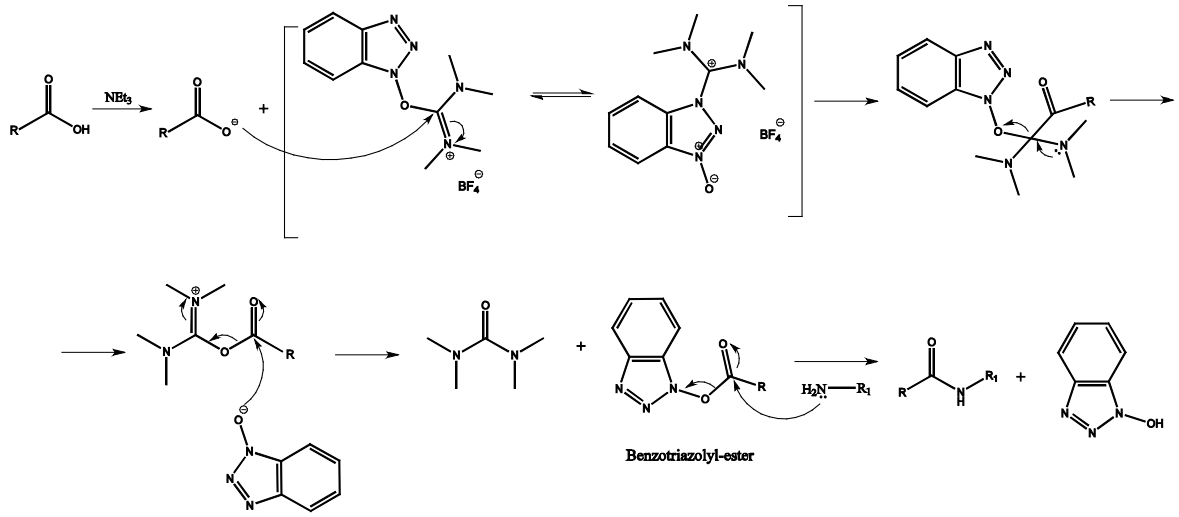


Figure 33. *Proposed mechanism for the amide bond formation through TBTU.*

Compound **8** was easily converted in compound **E2** through *N*-alkylation in presence of NaH 60%, LiBr and 4-fluorobenzyl chloride, heating at 65°C overnight. The derivative **E2** was purified by flash chromatography.

As reported in **Scheme 4**, compound **8** was treated with a solution of bromine in $CHCl_3$ to afford compound **9**, which was submitted to a *N*-alkylation reaction, in the same conditions previously described, to afford desired compound **E3**, which was purified by flash chromatography. This compound was subjected to a Suzuki cross-coupling reaction with the *p*-methoxyphenylboronic acid in toluene in the presence of tetrakis(trisphenylphosphine)palladium(0) as catalyst and anhydrous potassium carbonate as base, stirred at 110°C overnight to afford compound **E4**. Palladium(0) compound required in this cycle, $Pd(PPh_3)_4$, was prepared *in situ*

using triphenylphosphine (PPh_3) and palladium acetate ($Pd(OAc)_2$). Compound **E4** was purified by flash chromatography.

In order to obtain the 5-chlorinated-3-carboxamide derivative **10** (**Scheme 4**), compound **8** was treated with *N*-chlorosuccinimide using acetonitrile as solvent and refluxing overnight. Compound **10** was purified by flash chromatography. From this reaction, compound **11** was obtained in very low yields. The structure of compound **11** was verified by ^{1}H-NMR analysis since the protons of the chloromethyl group in 6-position are deshielded, therefore they display a resonance signal (singulet), that integrate for 2H, at low field.
Derivative **10** was alkylated with the same procedure previously reported, to afford desired compound **E5**, which was purified by flash chromatography.
Compound **11** was then alkylated with NaH 60%, LiBr and 4-fluorobenzyl chloride, heating at 65°C. Unfortunately, this reaction gave a mixture difficult to purify and the compound was not isolated (**Scheme 4**).

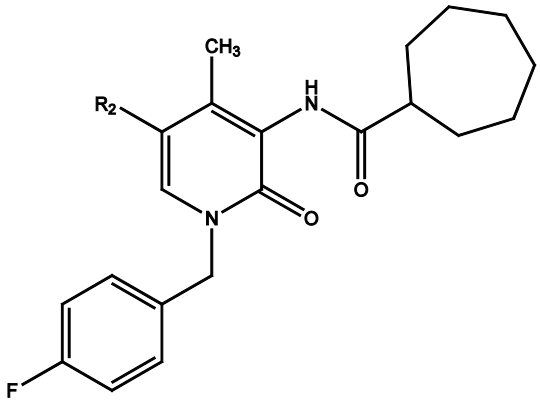
As reported in **Scheme 4**, the carboxamide **8** was treated with the fluorinating agent Selectfluor™ (1-chloromethyl-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane bis(tetrafluoroborate)[47, 48, 49, 50, 51] in refluxing acetonitrile for 12 hours, to afford the corresponding 5-fluorinated derivative **12**, which was purified by flash chromatography.[52]
Derivative **12** was alkylated with the same procedure previously used, to yield desired compound **E6**. In this case, the *O*-alkylated derivative **E7** was also isolated. The structures of compounds **E6-E7** were verified by ^{1}H-NMR analysis. The two isomers were distinguished since the methylene protons connected to the oxygen atom are more deshielded, therefore they display a resonance signal (singulet) at lower field than the two methylene protons bonded to the nitrogen atom.

Following the approach previously described for compound **10**, the 5-iodinated-3-carboxamide derivative was tried to be synthesized (**Scheme 4**).

Compound **8** was treated with *N*-iodosuccinimide, using acetonitrile as solvent, and refluxing overnight. Unfortunately, this reaction gave a mixture difficult to purify and the compound was not isolated.

Synthesis of N-(4-methyl-2-oxo-1,2-dihydropyridin-3-yl)cycloheptanecarboxamide derivatives F

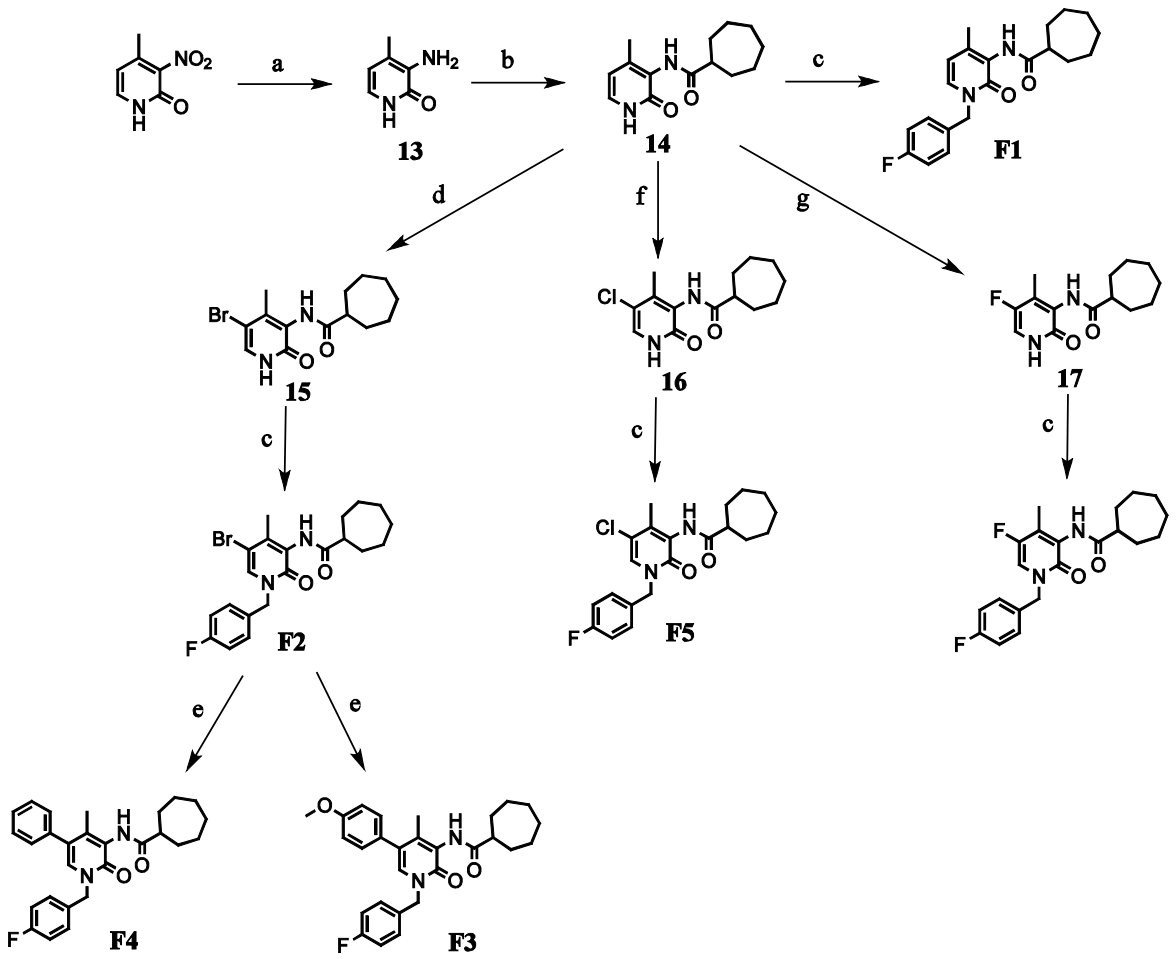
The synthesis of *N*-(4-methyl-2-oxo-1,2-dihydropyridin-3-yl)cycloheptanecarboxamide derivatives **F1-F5** (**Figure 34**) was planned following **Scheme 5**.



F1 R_2 = H;
F2 R_2 = Br;
F3 R_2 = *p*-Methoxyphenyl;
F4 R_2 = Phenyl;
F5 R_2 = Cl.

Figure 34. *Design of compounds **F1-F5**.*

Scheme 5. Synthetic route for compounds **F1-F5**



Reagents and conditions: a) H_2, Pd/(C), MeOH, rt, overnight; **b) 1)** Cycloheptancarboxilic acid, $SOCl_2$, 76°C, 3h **2)** Toluene, DMF, NEt_3, 0-32°C, 48h; **c) 1)** DMF, CsF, rt, 1h **2)** 4-fluorobenzyl chloride, 50°C, overnight; **d)** Br_2, $CHCl_3$, 30°C, overnight; **e)** Triphenylphosphine, $Pd(OAc)_2$, toluene, K_2CO_3, un/substituted-phenylboronic acid, 110°C, overnight; **f)** *N*-chlorosuccinimide, ACN, reflux, overnight; **g)** Selectfluor™, ACN, reflux, overnight.

Commercially available 2-hydroxy-4-methyl-3-nitropyridine was undergone to catalitic hydrogenation, to afford derivative **13**, which was treated with the coupling reagent TBTU at 0°C, in presence of cycloheptanecarboxilic acid and triethylamine. This last reaction failed, so the conditions were changed: the same reaction was performed increasing the temperature, but unsuccessfully.

The reason of this resistence is probably correlated to the steric hindrance of derivative **13**, due to the presence of the methyl group in position 4. For this reason, the amino group in position 3 of the pyridine ring was not probably able to attack the carbonyl moiety of benzotriazolyl-ester (**Figure 35**).

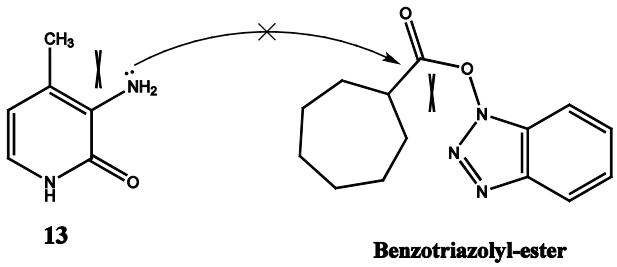


Figure 35. *Probable reason of the failure of carboxamide derivative synthesis via TBTU.*

Then the attention was focused on the pathway to synthesize the carboxamide derivative **14** through the involvement of acyl chloride, more reactive than benzotriazolyl-ester.

The reaction between the amine derivative **13** with the cycloheptanecarbonyl chloride in toluene, DMF and triethylamine at 0°C, and then at 32°C for 48 hours afforded compound **14**. The acyl chloride was obtained by the reaction between cycloheptanecarboxylic acid and $SOCl_2$ at 76°C for 3 hours. Derivative **14** was purified by flash chromatography.

Compound **14** was then *N*-alkylated for reaction with 4-fluorobenzyl chloride in presence of NaH 60%, LiBr and heating at 65°C overnight. Because the yield of this step was less than 10%, the reaction conditions were changed. Then, compound **14** was dissolved in DMF and treated with CsF for 1 hour before adding 4-fluorobenzyl chloride.[53] The reaction mixture was stirred at 50°C overnight, to afford desired compound **F1**, in 40% yield. Derivative **F1** was purified by flash chromatography.

Compound **14** was treated with a solution of bromine in $CHCl_3$, heating at 30°C to afford compound **15**, which was *N*-alkylated using CsF and 4-fluorobenzyl chloride, to obtain desired compound **F2**, which was purified by flash chromatography.
In order to obtain compounds **F3** and **F4**, derivative **F2** was submitted to a Suzuki cross-coupling reaction. Thus, toluene, triphenylphosphine and palladium acetate were mixed together to afford the catalyst tetrakis(trisphenylphosphine)palladium(0). After, potassium carbonate, compound **F2** and the suitable boronic acid (*p*-methoxyphenylboronic acid or phenylboronic acid) were added and heated at 110°C overnight to afford the desired compounds **F3** and **F4**, which were purified by flash chromatography.

In order to obtain the 5-chlorinated derivative **16** (**Scheme 5**), compound **14** was treated with *N*-chlorosuccinimide using acetonitrile as solvent, and refluxed overnight. Derivative **16** was then alkylated, following the same procedure used to obtain compounds **F1-F4**. Desired compound **F5** was purified by flash chromatography.

As reported in **Scheme 5**, the carboxamide **14** was treated with the fluorinating agent Selectfluor™ in refluxing acetonitrile for 12 hours, to afford the corresponding 5-fluorinated derivative **17**. Compound **17** was purified by flash chromatography. Derivative **17** was alkylated with the same procedure previously used. Unfortunately, the amount of the obtained product was not enough to be purified by flash chromatography.

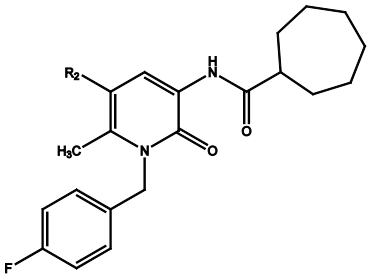
Results and Discussion

Compounds **E1-E7** and **F1-F5** are being tested for their activity and binding affinity (K_i values) towards both subtypes of CB receptors at the Institute of Biochemistry and Molecular Medicine of the University of Bern.

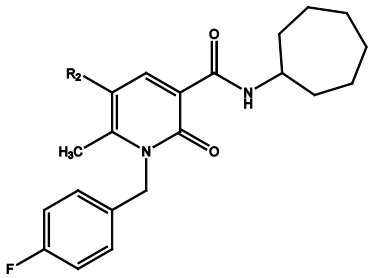
CB1 and CB2 receptor affinity

Until now, the preliminary screening of *N*-(6-methyl-2-oxo-1,2-dihydropyridin-3-yl)cycloheptanecarboxamide derivatives **E2**, **E3** and **E5** is available. The binding affinities of these compounds (K_i values) are evaluated in competitive radioligand displacement assays against [^{3}H]CP-55,940 using membrane preparations obtained in-house from stable transfected CHO-*h*CB_1 and CHO-*h*CB_2 cells. The results are summarized in **Table 7**, where are also indicated K_i values of 6-methyl-2-oxo-1,2-dihydropyridine-3-carboxamide derivative **C1**, **C4** and **C6**, previously synthesized in laboratory where I performed my thesis.

Table 7 Radioligand binding data of *N*-(6-methyl-2-oxo-1,2-dihydropyridin-3-yl)cycloheptane carboxamide derivatives **E2**, **E3** and **E5**, and 6-methyl-2-oxo-1,2-dihydropyridine-3-carboxamide derivatives **C1**, **C4** and **C6**



E



C

	R_2	K_i (nM)		
		CB_1 [a]	**CB_2 [b]**	**SI [c]**
C1	H	14.2	9.4	3.3
C4	Br	8.9	2.8	3.1
C6	Cl	4	0.6	6.8
E2	H		118.34	
E3	Br		129.86	
E5	Cl		62.58	

[a] Affinity of compounds for CB_1R was evaluated using membranes from CHO cells transfected and [^{3}H]CP-55,940.

[b] Affinity of compounds for CB_2R was evaluated using membranes from CHO cells transfected and [^{3}H]CP-55,940.

[c] Selectivity index for CB_2R calculated as $K_i(CB_1R)/K_i(CB_2R)$ ratio.

The preliminary screening on cannabinoid receptors of compounds **E** shows that these compounds possess interesting affinity for CB2 receptor, but they are less active than the corresponding derivatives **C**. Subsequently there is an increase in the K_i values for compounds **E**. These results indicate that the reverse amide group does not interact with the binding site of CB2 receptor in the same way of the amide. However the new compounds **E** and **F** need further investigations in order to check their activity on other targets of the ECS.

Experimental part

Commercially available reagents were purchased from Sigma Aldrich or Alpha Aesar and used without purification.

The structure of all compounds was verified by means of spectrometry ^{1}H-NMR and ^{13}C-NMR. The spectra of proton nuclear magnetic resonance were performed with a Bruker AVANCE III™ 400 spectrometer operating at 400 MHz and referred to the residual solvent. Chemical shift (δ) are reported in parts per million, while coupling constants (*J*) are expressed in Hertz (Hz).

Evaporation was performed under vacuum using a rotary evaporator.

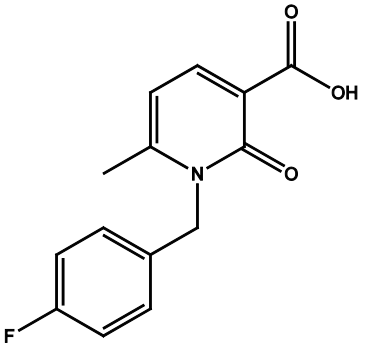
The system for isocratic flash chromatography includes glass chromatography columns and silica gel 60 Å (0.040-0.063 mm; MERK).

Reactions were monitored by TLC. The analytical TLC plates were performed using silica gel 60 F_{254} (MERCK) containing a fluorescent indicator; the various spots were highlighted by means of a UV lamp (254 nm).

Melting points were determined on a Kofler® hot stage apparatus and are uncorrected.

Catalitic hydrogenation was performed with CLAIND H_2 generator, type HG2200 (flow meter: 200 ml/min; outlet pressure: 1-6 bar; operating temperature: 5-40°C).

1-(4'-fluorobenzyl)-6-methyl-2-oxo-1,2-dihydropyridine-3-carboxylic acid (1)



To a solution of commercially available 6-methyl-2-oxo-1,2-dihydropyridine-3-carboxylic acid (0.200 g, 1.30 mmol) in DME (2.60 ml) and anhydrous DMF (0.650 ml), NaH 60% (0.0536 g, 1.34 mmol) was added portionwise under nitrogen at 0°C. LiBr (0.226 g, 2.60 mmol) was added 10 minutes later. The reaction contents were stirred 15 minutes at room temperature, 4-fluorobenzyl chloride (0.300 ml, 2.50 mmol) was added dropwise and the reaction was stirred at 65°C overnight.

The solvents were removed under reduced pressure.

The reaction mixture was dissolved in ethyl acetate and washed six times with brine. Then the organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure.

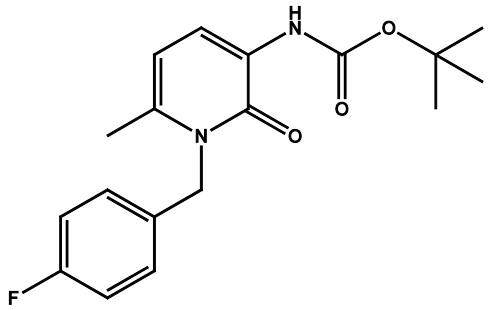
NaOH 60% (4.00 ml) was added to the crude residue obtained and the reaction contents were refluxed at 130°C for 4 hours.

The reaction mixture was extracted with $CHCl_3$ and concentrated HCl was added to the aqueous phase until pH=2. The aqueous phase was extracted with $CHCl_3$. Then the organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure to afford compound **1** (0.139 g, 0.528 mmol) as a yellow solid.

Yield: 41%

^{1}H-NMR: (DMSO) δ (ppm) 8.38 (d, *J*=7.6 Hz, 1H, H4 Py), 7.31-7.20 (m, 4H, Ar), 6.75 (d, *J*=7.6 Hz, 1H, H5 Py), 5.48 (s, 2H, benzylic CH_2), 2.53 (s, 3H, CH_3).

Tert-butyl [1-(4'-fluorobenzyl)-6-methyl-2-oxo-1,2-dihydropyridin-3-yl]-carbamate (2)



1-(4'-fluorobenzyl)-6-methyl-2-oxo-1,2-dihydropyridine-3-carboxylic acid **1** (0.215 g, 0.820 mmol) was dissolved in *tert*-butyl alcohol (14.4 ml) under heating and then diphenylphosphoryl azide (0.150 ml, 0.680 mmol) and potassium *tert*-butoxide (0.0766 g, 0.680 mmol) were added.

The reaction contents were refluxed at 100°C overnight.

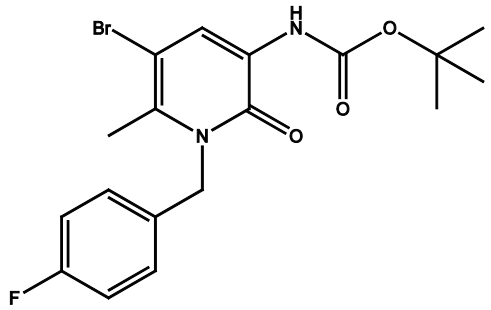
The reaction mixture was extracted with $CHCl_3$/water and then the organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure.

The brown oil obtained was purified by flash chromatography on silica gel using, as eluent, petroleum ether/ethyl acetate 8:2, to obtain compound **2** (0.235 g, 0.709 mmol) as a light brown oil.

Yield: 35%

^{1}H-NMR: ($CDCl_3$) δ (ppm) 7.91 (d, *J*=7.2 Hz, 1H, H4 Py), 7.61 (bs, 1H, NH amide), 7.12-7.09 (m, 2H, Ar), 7.01-7.97 (m, 2H, Ar), 6.08 (d, *J*=7.6 Hz, 1H, H5 Py), 5.32 (s, 2H, benzylic CH_2), 2.26 (s, 3H, CH_3), 1.50 (s, 9H, *tert*-butyl).

Tert-butyl [5-bromo-1-(4'-fluorobenzyl)-6-methyl-2-oxo-1,2-dihydropyridin-3-yl]-carbamate (3)



Tert-butyl [1-(4'-fluorobenzyl)-6-methyl-2-oxo-1,2-dihydropyridin-3-yl]-carbamate **2** (0.235 g, 0.708 mmol) was dissolved in $CHCl_3$ (1.18 ml) at room temperature.

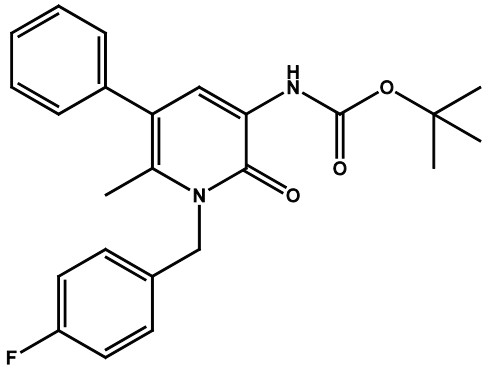
A solution of Br_2 (0.0400 ml, 0.708 mmol) in $CHCl_3$ (0.708 ml), previously prepared, was added dropwise to the first solution. The reaction contents were allowed to stir at room temperature overnight.

The reaction mixture was washed two times with a satured solution of $Na_2S_2O_3$. Then the organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated in vacuum to afford compound **3** (0.206 g, 0.502 mmol) as a light brown oil.

Yield: 71%

^{1}H-NMR: ($CDCl_3$) δ (ppm) 8.20 (bs, 1H, NH amide), 7.63 (s, 1H, H4 Py), 7.26-6.99 (m, 4H, Ar), 5.37 (s, 2H, benzylic CH_2), 2.41 (s, 3H, CH_3), 1.51 (s, 9H, *tert*-butyl).

Tert-butyl [1-(4'-fluorobenzyl)-6-methyl-2-oxo-5-phenyl-1,2-dihydropyridin-3-yl]-carbamate (4)



0.00368 g (0.0150 mmol, 0.0300 equivalents) of $Pd(OAc)_2$ were placed in a solution of 0.0197 g (0.0750 mmol) of PPh_3 in toluene in a vial under nitrogen.

After 10-15 minutes, phenylboronic acid (0.125 g, 1.00 mmol), K_2CO_3 (0.104 g, 0.753 mmol) and *tert*-butyl [5-bromo-1-(4'-fluorobenzyl)-6-methyl-2-oxo-1,2-dihydropyridin-3-yl]-carbamate **3** (0.206 g, 0.502 mmol) were added to reaction. Reaction contents were allowed to stir at 100°C overnight.

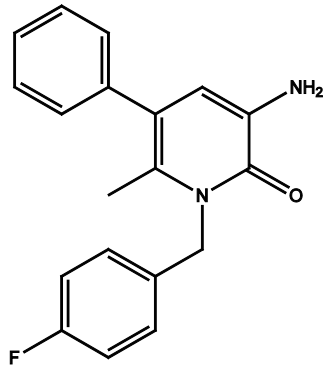
After the completion of the reaction, toluene was removed; reaction mixture was extracted with $CHCl_3$/water. Combined organic phase was dried over Na_2SO_4 and evaporated to obtain a brown oil.

The crude residue obtained was purified by flash chromatography on silica gel using, as eluent, petroleum ether/ethyl acetate 9:1 to give compound **4** (0.0835 g, 0.204 mmol) as a yellow oil.

Yield: 41%

^{1}H-NMR: ($CDCl_3$) δ (ppm) 8.03 (bs, 1H, NH amide), 7.68 (s, 1H, H4 Py), 7.38-7.31 (m, 2H, Ar), 7.31-7.28 (m, 1H, Ar), 7.24-7.15 (m, 2H, Ar), 7.14-7.13 (m, 2H, Ar), 7.03-6.99 (m, 2H, Ar), 5.43 (s, 2H, benzylic CH_2), 2.20 (s, 3H, CH_3), 1.49 (s, 9H, *tert*-butyl).

3-amino-1-(4'-fluorobenzyl)-6-methyl-5-phenylpyridin-2(1H)-one (5)



To a solution of *tert*-butyl [1-(4'-fluorobenzyl)-6-methyl-2-oxo-5-phenyl-1,2-dihydropyridin-3-yl]-carbamate **4** (0.0339 g, 0.0829 mmol) in ethyl acetate (1.39 ml), HCl 37% (0.270 ml, 3.32 mmol) was added.

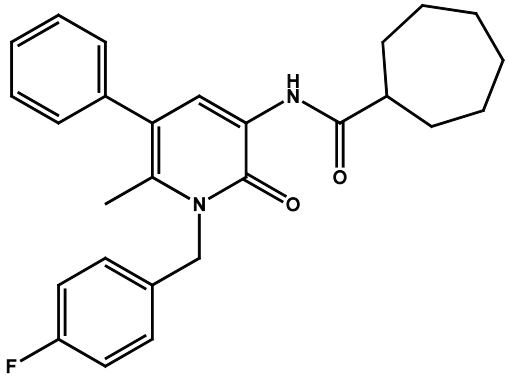
Reaction contents were allowed to stir at room temperature for 1 hour.

The reaction mixture was extracted with $CHCl_3$/water. Combined organic phase was dried over Na_2SO_4 and evaporated to afford compound **5** (0.0305 g, 0.0989 mmol) as a brown oil.

Yield: over 99%

^{1}H-NMR: ($CDCl_3$) δ (ppm) 7.39-6.98 (m, 9H, Ar), 6.61 (s, 1H, H4 Py), 5.42 (s, 2H, benzylic CH_2), 2.17 (s, 3H, CH_3).

N-[1-(4'-fluorobenzyl)-6-methyl-2-oxo-5-phenyl-1,2-dihydripyridin-3-yl]cycloheptanecarboxamide (E1)



Cycloheptanecarboxylic acid (0.0140 ml , 0.0989 mmol) was dissolved in $SOCl_2$ (0.105 ml, 1.44 mmol) in a vial under nitrogen. The solution was stirred at 76°C for 3 hours to allow the acyl chloride to generate.

In the meantime, 3-amino-1-(4'-fluorobenzyl)-6-methyl-5-phenylpyridin-2(1H)-one **5** (0.0305 g, 0.0989 mmol) was dissolved in toluene (4.58 ml) in a round bottom flask. After the dissolution, triethylamine (0.0300 ml, 0.198 mmol) was added and the temperature was lowered to 0°C.

After 3 hours, the solution in the vial was cooled to room temperature and then $SOCl_2$ was removed under nitrogen.

The acyl chloride formed was added dropwise, under nitrogen, to the solution in the the round bottom flask previously prepared and the reaction contents were allowed to stir at 25°C overnight.

After the completion of the reaction, toluene was removed; reaction mixture was extracted with ethyl acetate/water and washed with a satured solution of $NaHCO_3$. Combined organic phase was dried over Na_2SO_4 and evaporated to obtain a yellow oil.

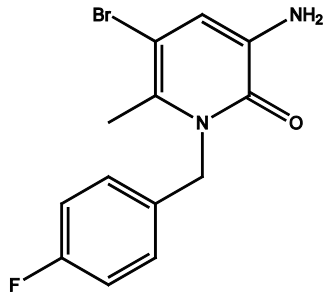
The yellow oil obtained was purified by flash chromatography on silica gel using, as eluent, petroleum ether/ethyl acetate 8:2 to give compound **E1** (0.00570 g, 0.0132 mmol) as a yellow oil.

Yield: 13%

^{1}H-NMR: ($CDCl_3$) δ (ppm) 8.44 (s, 1H, H4 Py), 8.37 (bs, 1H, NH amide), 7.37-7.00 (m, 9H, Ar), 5.43 (s, 2H, benzylic CH_2), 2.47-2.42 (m, 1H, CH cycloheptyl), 2.23 (s, 3H, CH_3), 1.98-1.1.94 (m, 2H, cycloheptyl), 1.80-1.70 (m, 4H, cycloheptyl), 1.58-1.46 (m, 6H, cycloheptyl).

^{13}C-NMR: ($CDCl_3$) δ (ppm) 176.33, 160.93, 158.08, 139.13, 131.86, 129.70, 128.39, 128.15, 128.07, 126.61, 125.09, 121.21, 115.96, 115.74, 48.32, 48.02, 31.54, 28.28, 26.46, 17.42.

3-Amino-5-bromo-1-(4'-fluorobenzyl)-6-methyl-2(1H)-one (6)



To a solution of *tert*-butyl [5-bromo-1-(4'-fluorobenzyl)-6-methyl-2-oxo-1,2-dihydropyridin-3-yl]-carbamate **3** (0.138 g, 0.336 mmol) in ethyl acetate (5.62 ml), HCl 37% (1.10 ml, 13.43 mmol) was added.

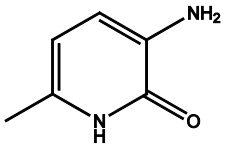
Reaction contents were allowed to stir at room temperature for 1 hour.

The reaction mixture was extracted with $CHCl_3$/water. Combined organic phase was dried over Na_2SO_4 and evaporated to give compound **6** (0.116 g, 0.373 mmol) as a brown oil.

Yield: over 99%

^{1}H-NMR: ($CDCl_3$) δ (ppm) 8.02 (s, 2H, NH_2), 7.13-7.09 (m, 2H, Ar), 7.02-6.97 (m, 2H, Ar), 6.83 (s, 1H, H4 Py), 5.37 (s, 2H, benzylic CH_2), 2.36 (s, 3H, CH_3).

2-Hydroxy-6-methyl-3-aminopyridine (7)



In a solution of commercial available 2-hydroxy-6-methyl-3-nitropyridine (1.00 g, 6.49 mmol) in methanol, Pd/C (0.100 g) was added.

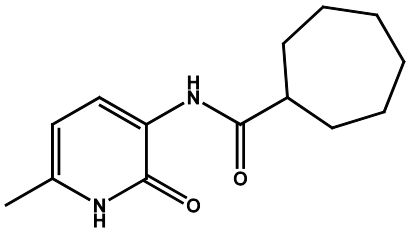
The reaction contents underwent catalitic hydrogenation (three alternated cycles of vacuum and hydrogen); after that, the reaction contents were allowed to stir under hydrogen at room temperature overnight.

The reaction mixture was filtered under vacuum using celite. The filtrate was evaporated under reduced pressure to afford compound **7** (0.823 g, 6.63 mmol) as an ocher solid.

Yield: over 99%

^{1}H-NMR: ($CDCl_3$) δ (ppm) 12.36 (bs, 1H, NH Py), 6.57 (d, *J*=7.2 Hz, 1H, H4 Py), 5.88 (d, *J*=7.2 Hz, 1H, H5 Py), 3.97 (bs, 2H, NH_2), 2.25 (s, 3H, CH_3).

N-(6-methyl-2-oxo-1,2-dihydripyridin-3-yl)cycloheptanecarboxamide (8)



A solution of cycloheptanecarboxylic acid (0.870 ml, 6.32 mmol) in anhydrous DMF (15.7 ml) was stirred in an ice-bath. TBTU (2.44 g, 7.59mmol) and triethylamine (2.40 ml, 17.7mmol) were added at 0°C. After 30 minutes, 2-hydroxy-3-amino-6-methylpyridine **7** (0.785 g, 6.32 mmol) was added and the reaction contents were stirred at 0°C for 30 minutes and then at room temperature overnight.

The solvent was removed under reduced pressure.

The reaction mixture was dissolved in $CHCl_3$ and washed ten times with water. The organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. The brown solid obtained was purified by flash chromatography on silica gel using, as eluent, ethyl acetate/petroleum ether 8:2, to obtain a light yellow solid. The solid was crystallized in hexane to afford compound **8** as a white crystal solid (1.29 g, 5.18 mmol).

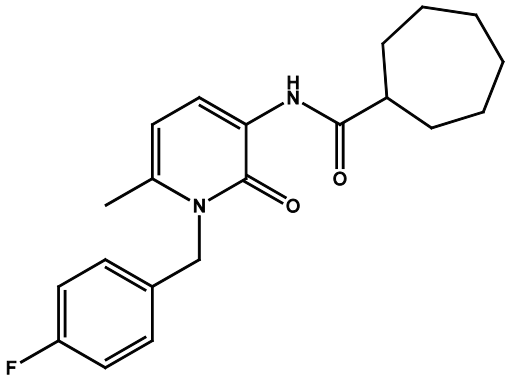
Yield: 82%

^{1}H-NMR: ($CDCl_3$) δ (ppm) 11.77 (bs, 1H, NH Py), 8.39 (d, 1H, *J*=7.6 Hz, H4 Py), 8.16 (bs, 1H, NH amide), 6.09 (d, 1H, *J*=7.6 Hz, H5 Py), 2.47-2.44 (m, 1H, CH cycloheptyl), 2.32 (s, 3H, CH_3), 2.01-1.95 (m, 2H, cycloheptyl), 1.83-1.71 (m, 4H, cycloheptyl), 1.62-1.49 (m, 6H, cycloheptyl).

Melting point: 182-185°C

Crystallization solvent: hexane

N-[1-(4'-fluorobenzyl)-6-methyl-2-oxo-1,2-dihydripyridin-3-yl]cycloheptanecarboxamide (E2)



To a solution of *N*-(6-methyl-2-oxo-1,2-dihydripyridin-3-yl)-cycloheptanecarboxamide **8** (0.100 g, 0.403 mmol) in DME (0.810 ml) and anhydrous DMF (0.200 ml) NaH 60% (0.0166 g, 0.415 mmol) was added portionwise at 0°C.

LiBr (0.0700 g, 0.806 mmol) was added 10 minutes later. The reaction contents were stirred 15 minutes at room temperature, 4-fluorobenzyl chloride (0.0930 ml, 0.778 mmol) was added dropwise and the reaction was stirred at 65°C overnight.

The solvents were removed under reduced pressure.

The reaction mixture was dissolved in $CHCl_3$ and washed three times with brine. Then the organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure.

The crude residue obtained was purified by flash chromatography on silica gel using, as eluent, petroleum ether/ethyl acetate (firstly 9:1 and then 8:2), to give compound **E2** (0.0377 g, 0.106 mmol) as a yellow oil.

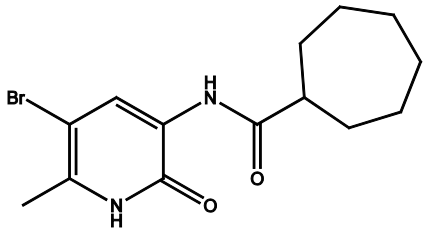
Yield: 26%

^{1}H-NMR: ($CDCl_3$) δ (ppm) 8.31 (bs, 1H, NH amide), 8.30 (d, 1H, *J*=7.6 Hz, H4 Py), 7.01-6.96 (m, 2H, Ar), 7.12-7.08 (m, 2H, Ar), 6.09 (d, 1H, *J*=7.6 Hz, H5 Py), 5.31 (s,

2H, benzylic CH_2), 2.45-2.40 (m, 1H, CH cycloheptyl), 2.26 (s, 3H, CH_3), 2.02-1.94 (m, 2H, cycloheptyl), 1.80-1.70 (m, 4H, cycloheptyl), 1.60-1.44 (m, 6H, cycloheptyl). ^{13}C-NMR: ($CDCl_3$) δ (ppm) 176.99, 161.60, 159.30, 138.52, 132.44, 132.41, 128.85, 128.77, 122.90, 116.59, 116.38, 107.88, 49.14, 48.18, 32.24, 28.87, 27.20, 20.70.

Crystallization solvent: hexane

N-(5-bromo-6-methyl-2-oxo-1,2-dihydripyridin-3-yl)cycloheptanecarboxamide (9)



N-(6-methyl-2-oxo-1,2-dihydripyridin-3-yl)-cycloheptanecarboxamide **8** (0.258 g, 1.04 mmol) was dissolved in $CHCl_3$ (1.73 ml) at room temperature.

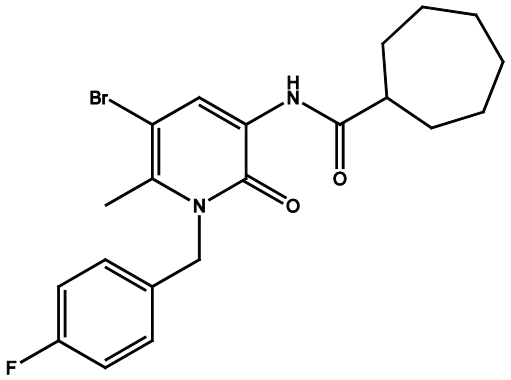
A solution of Br_2 (0.0530 ml, 1.04 mmol) in $CHCl_3$ (1.04 ml), previously prepared, was added dropwise to the first solution. The reaction contents were allowed to stir at room temperature overnight.

The reaction mixture was washed four times with a satured solution of $Na_2S_2O_3$. Then the organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated in vacuum to give compound **9** (0.304 g, 0.928 mmol) as a light yellow solid.

Yield: 89%

^{1}H-NMR: ($CDCl_3$) δ (ppm) 12.25 (bs, 1H, NH Py), 8.64 (s, 1H, H4 Py), 8.10 (s, 1H, NH amide), 2.50-2.41 (m, 1H, CH cycloheptyl), 2.01 (s, 3H, CH_3), 1.75-1.61 (m, 6H, cycloheptyl), 1.84-1.76 (m, 4H, cycloheptyl), 1.99-1.94 (m, 2H, cycloheptyl).

N-[5-bromo-1-(4'-fluorobenzyl)-6-methyl-2-oxo-1,2-dihydripyridin-3-yl]cycloheptanecarboxamide (E3)



To a solution of *N*-(5-bromo-6-methyl-2-oxo-1,2-dihydripyridin-3-yl)-cycloheptanecarboxamide **9** (0.279 g, 0.854 mmol) in DME (1.70 ml) and anhydrous DMF (0.400 ml) NaH 60% (0.0352 g, 0.879 mmol) was added portionwise at 0°C.

LiBr (0.148 g, 1.70 mmol) was added 10 minutes later. The reaction contents were stirred 15 minutes at room temperature, 4-fluorobenzyl chloride (0.200 ml, 1.65 mmol) was added dropwise and the reaction was stirred at 65°C overnight.

The solvents were removed under reduced pressure.

The reaction mixture was dissolved in $CHCl_3$ and washed three times with brine. Then the organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure.

The residue obtained was purified by flash chromatography on silica gel using, as eluent, petroleum ether/ethyl acetate (firstly 9:1 and then 8:2), to obtain a light yellow solid.

The solid was crystallized in hexane to afford compound **E3** as a white solid (0.150 g, 0.346 mmol).

Yield: 40%

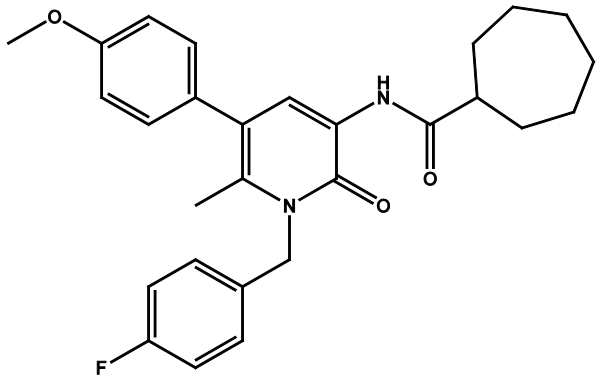
^{1}H-NMR: ($CDCl_3$) δ (ppm) 8.61 (s, 1H, H4 Py), 8.31 (bs, 1H, NH amide), 7.04-7.00 (m, 2H, Ar), 7.12-7.09 (m, 2H, Ar), 5.39 (s, 2H, benzylic CH_2), 2.46-2.40 (m, 1H, CH cycloheptyl), 2.42 (s, 3H, CH_3), 1.99-1.94 (m, 2H, cycloheptyl), 1.82-1.71 (m, 4H, cycloheptyl), 1.60-1.48 (m, 6H, cycloheptyl).

^{13}C-NMR: ($CDCl_3$) δ (ppm) 176.38, 161.01, 157.81, 135.69, 131.26, 131.23, 128.14, 128.06, 125.83, 116.06, 115.85, 101.58, 48.82, 48.28, 31.47, 28.23, 26.44, 19.54.

Melting point: 124-127°C

Crystallization solvent: hexane

N-[1-(4'-fluorobenzyl)-5-(4'-methoxyphenyl)-6-methyl-2-oxo-1,2-dihydripyridin-3-yl]cycloheptanecarboxamide (E4)



0.00254 g (0.0104 mmol) of $Pd(OAc)_2$ were placed in a solution of 0.0136 g (0.0519 mmol) of PPh_3 in toluene in a vial under nitrogen.

After 10-15 minutes, *p*-methoxyphenylboronic acid (0.107 g, 0.692 mmol), K_2CO_3 (0.0717 g, 0.519 mmol) and *N*-[5-bromo-1-(4'-fluorobenzyl)-6-methyl-2-oxo-1,2-dihydripyridin-3-yl]-cycloheptanecarboxamide **E3** (0.150 g, 0.346 mmol) were added to reaction. Reaction contents were allowed to stir at 110°C overnight.

After the completion of the reaction, toluene was removed; reaction mixture was extracted with $CHCl_3$/water. Combined organic phase was dried over Na_2SO_4 and evaporated to afford a brown oil.

The crude residue obtained was purified by flash chromatography on silica gel using, as eluent, petroleum ether/ethyl acetate 8:2 to give compound **E4** as a yellow oil.

Compound **E4** was crystallized in hexane to afford a yellow oil (0.0306 g, 0.0661 mmol).

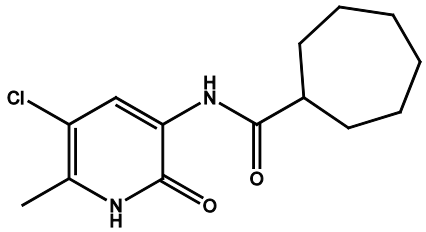
Yield: 19%

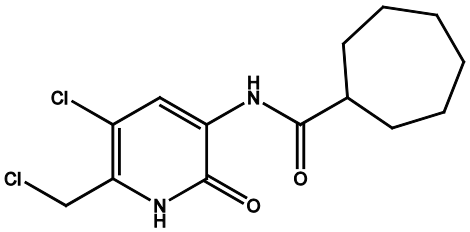
^{1}H-NMR: ($CDCl_3$) δ (ppm) 8.43 (s, 1H, H4 Py), 8.38 (bs, 1H, NH amide), 7.16-7.13 (m, 4H, Ar), 7.05-7.00 (m, 2H, Ar), 6.90-6.88 (m, 2H, Ar), 5.43 (s, 2H, benzylic CH_2), 3.83 (s, 3H, OCH_3), 2.48-2.43 (m, 1H, CH cycloheptyl), 2.23 (s, 3H, CH_3), 2.00-1.96 (m, 2H, cycloheptyl), 1.80-1.74 (m, 4H, cycloheptyl), 1.58-1.50 (m, 6H, cycloheptyl).

^{13}C-NMR: ($CDCl_3$) δ (ppm) 176.58, 161.16, 159.07, 158.28, 135.17, 132.15, 132.12, 131.03, 128.40, 128.32, 126.75, 125.62, 121.09, 116.19, 115.98, 114.05, 55.57, 48.58, 48.30, 31.80, 28.52, 26.72, 17.67.

Crystallization solvent: hexane

N-(5-chloro-6-methyl-2-oxo-1,2-dihydripyridin-3-yl)cycloheptanecarboxamide (10) and N-[5-chloro-6-(chloromethyl)-2-oxo-1,2-dihydropyridin-3-yl]cycloheptanecarboxamide (11)





In a suspension of *N*-(6-methyl-2-oxo-1,2-dihydripyridin-3-yl)-cycloheptanecarboxamide **8** (0.502 g, 2.02 mmol) and acetonitrile, a solution of *N*-chlorosuccinimide (0,270 g, 2.02 mmol) in acetonitrile was added.

Reaction contents were refluxed at 82°C overnight.

After the completion of the reaction, the solvent was removed under reduced pressure.

The mixture was quenched with water and extracted with ethyl acetate.

The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure.

The yellow solid obtained was purified by flash chromatography on silica gel using, as eluent, ethyl acetate/petroleum ether (firstly 5:5 and then 6:4), to give compound **10** as a white solid and compound **11** as a yellow solid.

Compound **10** was crystallized in hexane to afford a white solid (0.109 g, 0.385 mmol).

Yield (**10**): 19%

^{1}H-NMR (**10**): ($CDCl_3$) δ (ppm) 11.99 (bs, 1H, NH Py), 8.55 (s, 1H, H4 Py), 8.11 (bs, 1H, NH amide), 2.50-2.48 (m, 1H, CH cycloheptyl), 2.39 (s, 3H, CH_3), 2.01-1.96 (m, 2H, cycloheptyl), 1.85-1.81 (m, 4H, cycloheptyl), 1.79-1.54 (m, 6H, cycloheptyl).

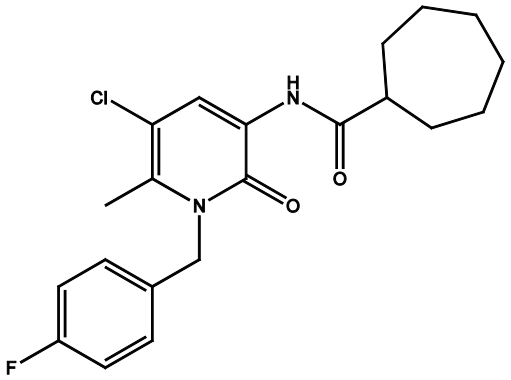
Melting point (**10**): 240-246°C

Crystallization solvent (**10**): hexane

Yield (**11**): 10%

^{1}H-NMR (**11**): ($CDCl_3$) δ (ppm) 8.53 (s, 1H, H4 Py), 8.26 (bs, 1H, NH amide), 4.63 (s, 2H, CH_2Cl), 2.54-2.49 (m, 1H, CH cycloheptyl), 2.01-1.95 (m, 2H, cycloheptyl), 1.84-1.71 (m, 4H, cycloheptyl), 1.65-1.54 (m, 6H, cycloheptyl).

N-[5-chloro-1-(4'-fluorobenzyl)-6-methyl-2-oxo-1,2-dihydripyridin-3-yl]cycloheptanecarboxamide (E5)



To a solution of *N*-(5-chloro-6-methyl-2-oxo-1,2-dihydripyridin-3-yl)-cycloheptanecarboxamide **10** (0.109 g, 0.385 mmol) in DME (0.770 ml) and anhydrous DMF (0.190 ml) NaH 60% (0.0159 g, 0.397 mmol) was added portionwise at 0°C.

LiBr (0.0669 g, 0.771 mmol) was added 10 minutes later. The reaction contents were stirred 15 minutes at room temperature, 4-fluorobenzyl chloride (0.0900 ml, 0.743 mmol) was added dropwise and the reaction was stirred at 65°C overnight.

The solvents were removed under reduced pressure.

The reaction mixture was dissolved in $CHCl_3$ and washed three times with brine. Then the organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure.

The brown oil obtained was purified by flash chromatography on silica gel using, as eluent, petroleum ether/ethyl acetate (firstly 9:1 and then 8:2), to give a brown solid.

The solid was crystallized in hexane to afford compound **E5** as a white solid (0.0537 g, 0.137 mmol).

Yield: 36%

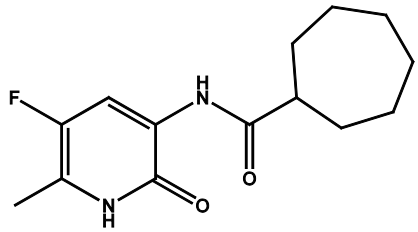
^{1}H-NMR: ($CDCl_3$) δ (ppm) 8.50 (s, 1H, H4 Py), 8.33 (bs, 1H, NH amide), 7.12-7.09 (m, 2H, Ar), 7.04-7.00 (m, 2H, Ar), 5.37 (s, 2H, benzylic CH_2), 2.46-2.42 (m, 1H, CH cycloheptyl), 2.38 (s, 3H, CH_3), 2.00-1.94 (m, 2H, cycloheptyl), 1.81-1.71 (m, 4H, cycloheptyl), 1.61-1.48 (m, 6H, cycloheptyl).

^{13}C-NMR: ($CDCl_3$) δ (ppm) 176.39, 161.01, 157.56, 134.35, 131.27, 131.24, 128.15, 128.07, 123.48, 116.06, 115.84, 113.35, 48.50, 48.30, 31.47, 28.21, 26.44, 16.68.

Melting point: 116-119°C

Crystallization solvent: hexane

N-(5-fluoro-6-methyl-2-oxo-1,2-dihydripyridin-3-yl)cycloheptanecarboxamide (12)



To a suspension of *N*-(6-methyl-2-oxo-1,2-dihydripyridin-3-yl)-cycloheptanecarboxamide **8** (0.700 g, 2.82 mmol) and acetonitrile, Selectfluor™ (1.199 g, 3.385 mmol) was added at room temperature.

Reaction contents were refluxed at 89°C for 12 hours.

After the completion of the reaction, the solvent was removed under reduced pressure.

The mixture was diluted in ethyl acetate and washed three times with water.

The organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure.

The black solid obtained was purified by flash chromatography on silica gel using, as eluent, petroleum ether/ethyl acetate 5:5, to give compound **12** (0.0522 g, 0.196 mmol) as a black solid.

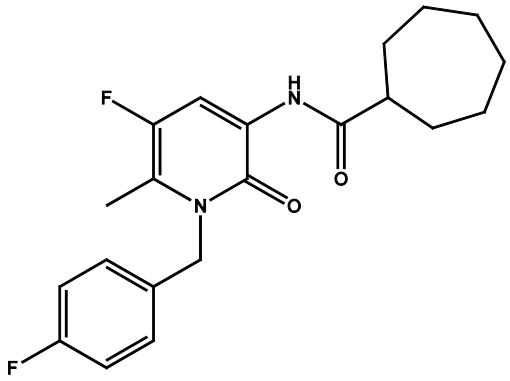
Yield: 7%

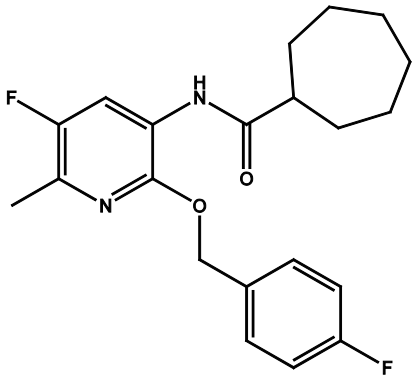
^{1}H-NMR: ($CDCl_3$) δ (ppm) 8.48 (d, 1H, *J*=10.4 Hz, H4 Py), 8.16 (bs, 1H, NH amide), 2.51-2.46 (m, 1H, CH cycloheptyl), 2.30 (d, 3H, *J*=2.8 Hz, CH_3), 2.01-1.95 (m, 2H, cycloheptyl), 1.81-1.75 (m, 4H, cycloheptyl), 1.62-1.53 (m, 6H, cycloheptyl).

Melting point: 92-98°C

Crystallization solvent: hexane

N-[5-fluoro-1-(4'-fluorobenzyl)-6-methyl-2-oxo-1,2-dihydripyridin-3-yl]cycloheptanecarboxamide (E6) and N-[5-fluoro-2-((4'-fluorobenzyl)oxy)-6-methylpyridin-3-yl]cycloheptanecarboxamide (E7)





To a solution of *N*-(5-fluoro-6-methyl-2-oxo-1,2-dihydripyridin-3-yl)-cycloheptanecarboxamide **12** (0.0563 g, 0.211 mmol) in DME (0.420 ml) and anhydrous DMF (0.100 ml) NaH 60% (0.00871 g, 0.218 mmol) was added portionwise at 0°C.

LiBr (0.0367 g, 0.422 mmol) was added 10 minutes later. The reaction contents were stirred 15 minutes at room temperature, 4-fluorobenzyl chloride (0.0500 ml, 0.408 mmol) was added dropwise and the reaction was stirred at 65°C overnight.

The solvents were removed under reduced pressure.

The reaction mixture was dissolved in $CHCl_3$ and washed three times with brine. Then the organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure.

The black oil obtained was purified by flash chromatography on silica gel using, as eluent, petroleum ether/ethyl acetate (firstly 9:1 and then 8:2), to give compound **E6** as a brown oil and compound **E7** as a white solid.

Compounds **E6** and **E7** were crystallized in distilled petroleum ether to afford a yellow oil (0.0191 g, 0.0510 mmol) and a white solid (0.0177 g, 0.0473 mmol) respectively.

Yield (**E6**): 24%

^{1}H-NMR (**E6**): ($CDCl_3$) δ (ppm) 8.43 (d, 1H, *J*=10.4 Hz, H4 Py), 8.40 (bs, 1H, NH amide), 7.14-7.11 (m, 2H, Ar), 7.04-7.00 (m, 2H, Ar), 5.32 (s, 2H, benzylic CH_2), 2.47-2.43 (m, 1H, CH cycloheptyl), 2.24 (d, 3H, *J*=3.2 Hz, CH_3 Py), 2.05-1.95 (m, 2H, cycloheptyl), 1.80-1.73 (m, 4H, cycloheptyl), 1.59-1.57 (m, 6H, cycloheptyl).

^{13}C-NMR (**E6**): ($CDCl_3$) δ (ppm) 176.54, 161.11, 156.74, 144.40, 131.49, 131.46, 128.34, 128.26, 127.16, 123.26, 116.12, 115.91, 48.45, 47.78, 31.56, 28.28, 26.56, 11.78.

Crystallization solvent (**E6**): distilled petroleum ether

Yield (**E7**): 22%

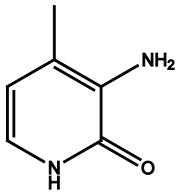
^{1}H-NMR (**E7**): ($CDCl_3$) δ (ppm) 8.43 (d, 1H, *J*=10.4 Hz, H4 Py), 7.52 (bs, 1H, NH amide), 7.44-7.41 (m, 2H, Ar), 7.10-7.06 (m, 2H, Ar), 5.38 (s, 2H, benzylic CH_2), 2.40-2.38 (m, 1H, CH cycloheptyl), 2.35 (d, 3H, *J*=3.2 Hz, CH_3 Py), 1.97-1.91 (m, 2H, cycloheptyl), 1.79-1.68 (m, 4H, cycloheptyl), 1.59-1.56 (m, 6H, cycloheptyl).

^{13}C-NMR (**E7**): ($CDCl_3$) δ (ppm) 176.01, 161.64, 154.26, 151.83, 147.31, 133.11, 130.51, 130.43, 121.20, 121.14, 115.63, 115.60, 67.79, 48.49, 31.74, 28.55, 28.50, 26.58, 17.25.

Melting point (**E7**): 90-103°C

Crystallization solvent (**E7**): distilled petroleum ether

2-Hydroxy-4-methyl-3-aminopyridine (13)



In a solution of commercial available 2-hydroxy-4-methyl-3-nitropyridine (1.00 g, 6.49 mmol) in methanol, Pd/C (0.100 g) was added.

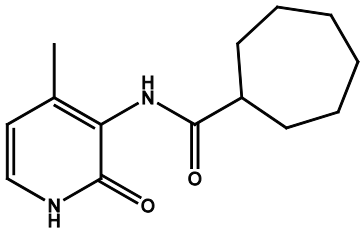
The reaction contents underwent catalitic hydrogenation (three alternated cycles of vacuum and hydrogen); after that, the reaction contents were allowed to stir under hydrogen at room temperature overnight.

The reaction mixture was filtered under vacuum using celite and the filtered was evaporated under reduced pressure to afford compound **15** (0.807 g, 6.50 mmol) as a brown solid.

Yield: over 99%

^{1}H-NMR: ($CDCl_3$) δ (ppm) 12.20 (bs, 1H, NH Py), 6.78 (d, *J*=6.8 Hz, 1H, H6 Py), 6.09 (d, *J*=6.8 Hz, 1H, H5 Py), 4.03 (bs, 2H, NH_2), 2.10 (s, 3H, CH_3).

N-(4-methyl-2-oxo-1,2-dihydripyridin-3-yl)cycloheptanecarboxamide (14)



Cycloheptanecarboxylic acid (0.700 ml , 4.99 mmol) was dissolved in $SOCl_2$ (2.60 ml, 36.5 mmol) in a vial under nitrogen. The solution was stirred at 76°C for 3 hours to allow the acyl chloride to generate.

In the meantime, 2-hydroxy-4-methyl-3-aminopyridine **15** (0.310 g, 2.49 mmol) was dissolved in toluene (46.5 ml) and anhydrous DMF (6.20 ml) in a round bottom flask. After the dissolution, triethylamine (0.700 ml, 4.99 mmol) was added and the temperature was lowered to 0°C.

After 3 hours, the solution in the vial was cooled to room temperature and then $SOCl_2$ was removed under nitrogen.

The acyl chloride formed was added dropwise, under nitrogen, to the solution in the the round bottom flask previously prepared and the reaction contents were allowed to stir at 32°C for 48 hours.

The solvents were removed under reduced pressure.

The reaction mixture was dissolved in $CHCl_3$ and washed three times with water. Then the organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure.

The brown solid obtained was purified by flash chromatography on silica gel using, as eluent, ethyl acetate with 2% acetic acid, to give compound **16** as an ocher solid. Compound **16** was triturated in hexane to afford a white solid (0.371 g, 1.49 mmol).

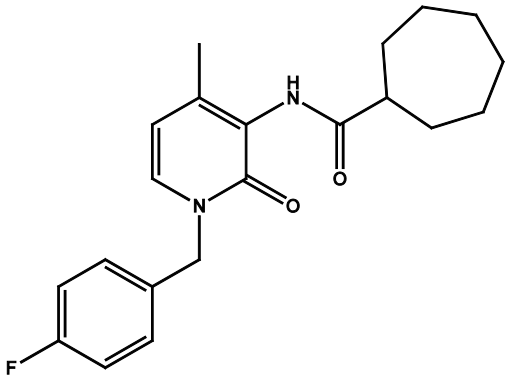
Yield: 60%

^{1}H-NMR: ($CDCl_3$) δ (ppm) 7.62 (bs, 1H, NH amide), 7.10 (d, 1H, *J*=6.4 Hz, H6 Py), 6.26 (d, 1H, *J*=6.8 Hz, H5 Py), 2.53-2.48 (m, 1H, CH cycloheptyl), 2.16 (s, 3H, CH_3), 2.02-1.98 (m, 2H, cycloheptyl), 1.81-1.71 (m, 4H, cycloheptyl), 1.59-1.53 (m, 6H, cycloheptyl).

Decomposition temperature: 150°C

Crystallization solvent: hexane

N-[1-(4'-fluorobenzyl)-4-methyl-2-oxo-1,2-dihydripyridin-3-yl]cycloheptanecarboxamide (F1)



To a solution of *N*-(4-methyl-2-oxo-1,2-dihydripyridin-3-yl)-cycloheptanecarboxamide **16** (0.0247 g, 0.0995 mmol) in anhydrous DMF (0.300 ml), CsF (0.0454 g, 0.298 mmol) was added.

The reaction contents were allowed to stir at room temperature for 1 hour; 4-fluorobenzyl chloride (0.0400 ml, 0.298 mmol) was added dropwise and the reaction was stirred at 50°C overnight.

The solvent was removed under reduced pressure.

The reaction mixture was treated with ice-water and then extracted with CH_2Cl_2. The organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure.

The brown oil obtained was purified by flash chromatography on silica gel using, as eluent, petroleum ether/ethyl acetate 5:5, to obtain compound **F1** (0.0140 g, 0.0393 mmol) as a white solid.

Yield: 40%

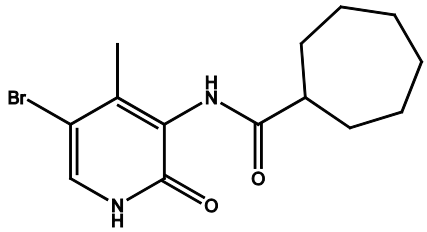
^{1}H-NMR: ($CDCl_3$) δ (ppm) 7.43 (bs, 1H, NH amide), 7.28-7.25 (m, 2H, Ar), 7.04 (d, 1H, *J*=2.2 Hz, H6 Py), 7.03-7.00 (m, 2H, Ar), 6.10 (d, 1H, *J*=7.2 Hz, H5 Py), 5.07 (s,

2H, benzylic CH_2), 2.52-2.47 (m, 1H, CH cycloheptyl), 2.12 (s, 3H, CH_3), 2.06-2.00 (m, 2H, cycloheptyl), 1.83-1.71 (m, 4H, cycloheptyl), 1.63-1.47 (m, 6H, cycloheptyl). ^{13}C-NMR: ($CDCl_3$) δ (ppm) 176.22, 161.59, 159.72, 142.57, 132.22, 131.84, 130.23, 130.14, 126.02, 116.23, 116.01, 110.38, 52.15, 48.20, 32.09, 28.43, 26.94, 20.00.

Melting point: 160-165°C

Crystallization solvent: hexane

N-(5-bromo-4-methyl-2-oxo-1,2-dihydripyridin-3-yl)cycloheptanecarboxamide (15)



N-(4-methyl-2-oxo-1,2-dihydripyridin-3-yl)-cycloheptanecarboxamide **16** (0.184 g, 0.743 mmol) was dissolved in $CHCl_3$ (1.20 ml) at room temperature.

A solution of Br_2 (0.0400 ml, 0.743 mmol) in $CHCl_3$ (0.700 ml), previously prepared, was added dropwise to the first solution. The reaction contents were allowed to stir at 30°C for 24 hours.

The reaction mixture was washed three times with a satured solution of $Na_2S_2O_3$. Then the organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated in vacuum to give compound **17** as a light yellow solid.

Compound **17** was triturated in hexane to afford a white solid (0.207 g, 0.634 mmol).

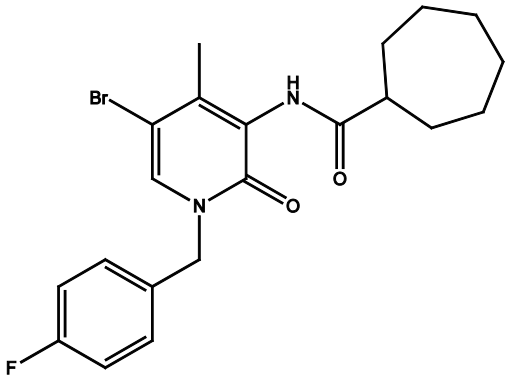
Yield: 85%

^{1}H-NMR: ($CDCl_3$) δ (ppm) 7.40 (bs, 1H, NH amide), 7.39 (s, 1H, H4 Py), 2.53-2.51 (m, 1H, CH cycloheptyl), 2.20 (s, 3H, CH_3), 2.05-2.01 (m, 2H, cycloheptyl), 1.83-1.75 (m, 4H, cycloheptyl), 1.61-1.55 (m, 6H, cycloheptyl).

Melting point: 217-219°C

Crystallization solvent: hexane

N-[5-bromo-1-(4'-fluorobenzyl)-4-methyl-2-oxo-1,2-dihydripyridin-3-yl]cycloheptanecarboxamide (F2)



To a solution of *N*-(5-bromo-4-methyl-2-oxo-1,2-dihydripyridin-3-yl)-cycloheptanecarboxamide **17** (0.154 g, 0.470 mmol) in anhydrous DMF (1.40 ml), CsF (0.214 g, 1.41 mmol) was added.

The reaction contents were allowed to stir at room temperature for 1 hour; 4-fluorobenzyl chloride (0.170 ml, 1.41 mmol) was added dropwise and the reaction was stirred at 50°C overnight.

The solvent was removed under reduced pressure.

The reaction mixture was treated with ice-water and then extracted with CH_2Cl_2. The organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure.

The brown solid obtained was purified by flash chromatography on silica gel using, as eluent, petroleum ether/ethyl acetate 7:3, to obtain a yellow solid.

The solid was crystallized in hexane to afford compound **F2** as a light yellow solid (0.119 g, 0.272 mmol).

Yield: 58%

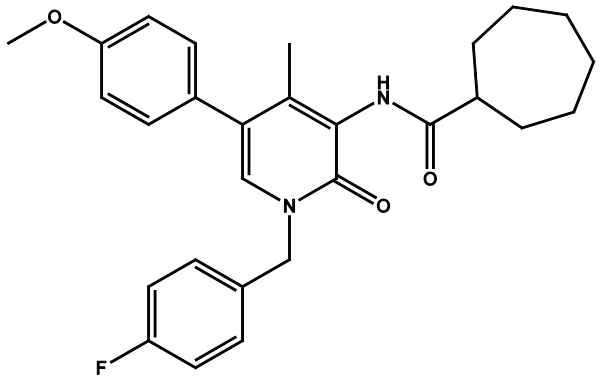
^{1}H-NMR: ($CDCl_3$) δ (ppm) 7.48 (bs, 1H, NH amide), 7.33 (s, 1H, H4 Py), 7.30-7.27 (m, 2H, Ar), 7.06-7.02 (m, 2H, Ar), 5.05 (s, 2H, benzylic CH_2), 2.51-2.48 (m, 1H, CH cycloheptyl), 2.16 (s, 3H, CH_3), 2.06-2.00 (m, 2H, cycloheptyl), 1.82-1.71 (m, 4H, cycloheptyl), 1.62-1.49 (m, 6H, cycloheptyl).

^{13}C-NMR: ($CDCl_3$) δ (ppm) 176.23, 161.68, 158.56, 142.22, 132.24, 131.50, 130.39, 130.30, 126.75, 116.37, 116.15, 104.22, 52.20, 48.08, 31.96, 28.37, 26.85, 20.81.

Melting point: 167-170°C

Crystallization solvent: hexane

N-[1-(4'-fluorobenzyl)-5-(4'-methoxyphenyl)-4-methyl-2-oxo-1,2-dihydripyridin-3-yl]cycloheptanecarboxamide (F3)



0.00165 g (0.00673 mmol) of $Pd(OAc)_2$ were placed in a solution of 0.00883 g (0.0337 mmol) of PPh_3 in toluene in a vial under nitrogen.

After 10-15 minutes, *p*-methoxyphenylboronic acid (0.0696 g, 0.449 mmol), K_2CO_3 (0.0465 g, 0.337 mmol) and *N*-[5-bromo-1-(4'-fluorobenzyl)-6-methyl-2-oxo-1,2-dihydripyridin-3-yl]-cycloheptanecarboxamide **F2** (0.0977 g, 0.224 mmol) were added to reaction. Reaction contents were allowed to stir at 110°C overnight.

After the completion of the reaction, the mixture was filtered under vacuum using celite and then toluene was removed. Reaction mixture was extracted with $CHCl_3$/water. Combined organic phase was dried over Na_2SO_4 and evaporated to afford a brown oil.

The crude residue obtained was purified by flash chromatography on silica gel using, as eluent, petroleum ether/ethyl acetate 5:5 to give compound **F3** as a yellow oil.

Compound **F3** was crystallized in hexane to afford a yellow oil (0.0748 g, 0.162 mmol).

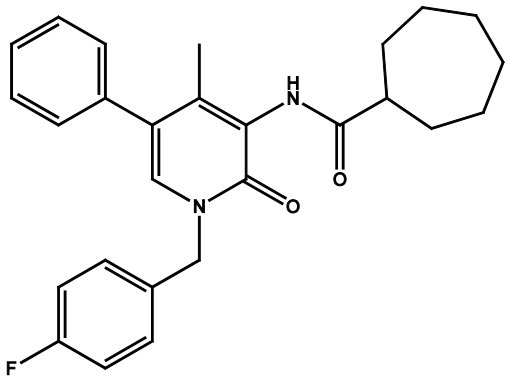
Yield: 72%

^{1}H-NMR: ($CDCl_3$) δ (ppm) 7.54 (bs, 1H, NH amide), 7.32-7.28 (m, 2H, Ar), 7.17-7.15 (m, 2H, Ar), 7.04-7.00 (m, 3H, Ar, H6 Py), 6.92-6.90 (m, 2H, Ar), 5.11 (s, 2H, benzylic CH_2), 3.83 (s, 3H, OCH_3), 2.54-2.50 (m, 1H, CH cycloheptyl), 2.12-2.01 (m, 2H, cycloheptyl), 1.98 (s, 3H, CH_3), 1.83-1.74 (m, 4H, cycloheptyl), 1.59-1.53 (m, 6H, cycloheptyl).

^{13}C-NMR: ($CDCl_3$) δ (ppm) 176.12, 161.33, 159.22, 158.66, 142.22, 135.86, 131.91, 130.78, 130.08, 130.00, 129.02, 125.82, 123.43, 116.00, 115.78, 113.92, 55.39, 51.97, 47.86, 31.85, 28.23, 26.67, 18.47.

Crystallization solvent: hexane

N-[1-(4'-fluorobenzyl)-4-methyl-2-oxo-5-phenyl-1,2-dihydripyridin-3-yl]cycloheptanecarboxamide (F4)



0.00220 g (0.00901 mmol) of $Pd(OAc)_2$ were placed in a solution of 0.0118 g (0.0450 mmol) of PPh_3 in toluene in a vial under nitrogen.

After 10-15 minutes, phenylboronic acid (0.0930 g, 0.600 mmol), K_2CO_3 (0.0622 g, 0.450 mmol) and *N*-[5-bromo-1-(4'-fluorobenzyl)-4-methyl-2-oxo-1,2-dihydripyridin-3-yl]-cycloheptanecarboxamide **F2** (0.131 g, 0.300 mmol) were added to reaction. Reaction contents were allowed to stir at 110°C overnight.

After the completion of the reaction, the mixture was filtered under vacuum using celite and then toluene was removed. Reaction mixture was extracted with $CHCl_3$/water. Combined organic phase was dried over Na_2SO_4 and evaporated to afford a yellow oil.

The crude residue obtained was purified by flash chromatography on silica gel using, as eluent, petroleum ether/ethyl acetate 6:4 to give compound **F4** as a yellow oil.

Compound **F4** was crystallized in hexane to afford a white crystal solid (0.0589 g, 0.136 mmol).

Yield: 45%

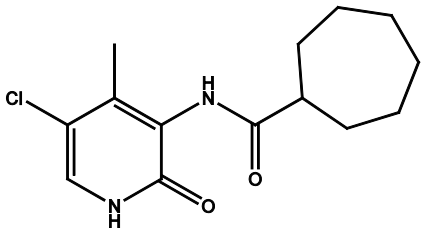
^{1}H-NMR: ($CDCl_3$) δ (ppm) 7.54 (bs, 1H, NH amide), 7.52-7.24 (m, 7H, Ar), 7.06-7.01 (m, 7H, Ar, H6 Py), 5.13 (s, 2H, benzylic CH_2), 2.54 (m, 1H, CH cycloheptyl), 2.07-2.02 (m, 2H, cycloheptyl), 2.00 (s, 3H, CH_3), 1.82-1.75 (m, 4H, cycloheptyl), 1.60-1.29 (m, 6H, cycloheptyl).

^{13}C-NMR: ($CDCl_3$) δ (ppm) 176.05, 161.35, 158.72, 141.85, 136.78, 131.94, 131.91, 131.00, 130.08, 130.00, 129.67, 128.52, 127.72, 126.02, 123.77, 115.99, 51.98, 47.81, 31.85, 28.25, 26.67, 18.41.

Melting point: 143-146°C

Crystallization solvent: hexane

N-(5-chloro-4-methyl-2-oxo-1,2-dihydripyridin-3-yl)cycloheptanecarboxamide (16)



In a suspension of *N*-(4-methyl-2-oxo-1,2-dihydripyridin-3-yl)-cycloheptanecarboxamide **16** (0.189 g, 0.762 mmol) and acetonitrile, *N*-chlorosuccinimide (0.102 g, 0.762 mmol) was added. Reaction contents were refluxed at 89°C overnight. After the completion of the reaction, the solvent was removed under reduced pressure.

The mixture was quenched with water and extracted with ethyl acetate.

The organic phase was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The yellow solid obtained was purified by flash chromatography on silica gel using, as eluent, ethyl acetate/petroleum ether 7:3 with 1% acetic acid, to give compound **18** as a yellow solid.

Compound **18** was triturated in hexane to afford a white solid (0.104 g, 0.368 mmol).

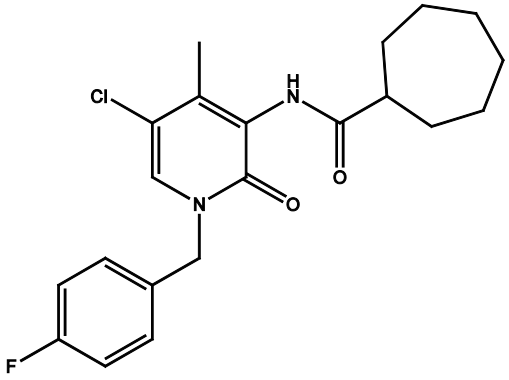
Yield: 48%

^{1}H-NMR: ($CDCl_3$) δ (ppm) 8.73 (bs, 1H, NH Py), 7.52 (s, 1H, H6 Py), 7.29 (bs, 1H, NH amide), 2.54 (m, 1H, CH cycloheptyl), 2.18 (s, 3H, CH_3), 2.04-2.01 (m, 2H, cycloheptyl), 1.83-1.73 (m, 4H, cycloheptyl), 1.66-1.49 (m, 6H, cycloheptyl).

Decomposition temperature: 200°C

Crystallization solvent: hexane

N-[5-chloro-1-(4'-fluorobenzyl)-4-methyl-2-oxo-1,2-dihydripyridin-3-yl]cycloheptanecarboxamide (F5)



To a solution of *N*-(5-chloro-4-methyl-2-oxo-1,2-dihydripyridin-3-yl)-cycloheptanecarboxamide **18** (0.104 g, 0.368 mmol) in anhydrous DMF (1.10 ml), CsF (0.168 g, 1.10 mmol) was added.

The reaction contents were allowed to stir at room temperature for 1 hour; 4-fluorobenzyl chloride (0.132 ml, 1.10 mmol) was added dropwise and the reaction was stirred at 50°C overnight.

The solvent was removed under reduced pressure.

The reaction mixture was treated with ice-water and then extracted with CH_2Cl_2. The organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure.

The brown oil obtained was purified by flash chromatography on silica gel using, as eluent, petroleum ether/ethyl acetate (firstly 7:3 and then 5:5), to obtain compound **F5** as a light yellow solid.

Compound **F5** was triturated in hexane to afford a white solid (0.0559 g, 0.143 mmol).

Yield: 39%

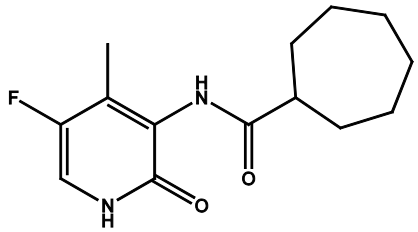
^{1}H-NMR: ($CDCl_3$) δ (ppm) 7.49 (bs, 1H, NH amide), 7.31-7.28 (m, 2H, Ar), 7.22 (s, 1H, H6 Py), 7.07-7.03 (m, 2H, Ar), 5.07 (s, 2H, benzylic CH_2), 2.52-2.49 (m, 1H, CH cycloheptyl), 2.15 (s, 3H, CH_3), 2.06-2.00 (m, 2H, cycloheptyl), 1.83-1.72 (m, 4H, cycloheptyl), 1.62-1.50 (m, 6H, cycloheptyl).

^{13}C-NMR: ($CDCl_3$) δ (ppm) 176.04, 161.47, 158.21, 141.14, 131.32, 131.28, 130.19, 130.11, 129.60, 126.61, 116.13, 115.92, 115.73, 51.99, 47.81, 31.75, 31.64, 28.26, 28.17, 26.64, 17.83.

Melting point: 197-199°C

Crystallization solvent: hexane

N-(5-fluoro-4-methyl-2-oxo-1,2-dihydripyridin-3-yl)cycloheptanecarboxamide (17)



To a suspension of *N*-(4-methyl-2-oxo-1,2-dihydripyridin-3-yl)cycloheptanecarboxamide **14** (0.370 g, 1.49 mmol) and acetonitrile, Selectfluor™ (0.634 g, 1.79 mmol) was added at room temperature.

Reaction contents were refluxed at 89°C for 12 hours.

After the completion of the reaction, the solvent was removed under reduced pressure.

The mixture was diluted in ethyl acetate and washed three times with water.

The organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure.

The brown solid obtained was purified by flash chromatography on silica gel using, as eluent, petroleum ether/ethyl acetate 6:4, to give compound **17** (0.0280 g, 0.105 mmol) as a yellow solid.

Yield: 7%

^{1}H-NMR: ($CDCl_3$) δ (ppm) 8.49 (bs, 1H, NH amide), 7.72 (s, 1H, H4 Py), 2.57 (m, 1H, CH cycloheptyl), 2.04 (s, 3H, CH_3), 2.00 (m, 2H, cycloheptyl), 1.80-1.78 (m, 4H, cycloheptyl), 1.62-1.52 (m, 6H, cycloheptyl).

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