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Tesi di Laurea:

DESIGN AND SYNTHESIS OF 2-OXO-1,2-DIHYDROPYRIDINE-3-CARBOXAMIDE DERIVATIVES AS NEW MODULATORS OF ENDOCANNABINOID SYSTEM.

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General introduction

Cannabis sativa L. (cannabis) is an annual dioecious plant belonging to the family Cannabaceae. Cannabis has a long history of human use as a medicinal plant, intoxicant, and ritual drug.[1] Today most nations' worldwide regard cannabis as an illegal drug of abuse. Despite the abuse, potential of cannabis research into its chemistry and pharmacology has demonstrated that it also has medical properties. Chemical analysis of cannabis in the 1940s and 1960s led to the discovery of a unique group of terpeno-phenolic secondary metabolites, known as cannabinoids, of which trans-(Δ)-D-9-tetrahydrocannabinol (Δ^9-THC) was shown to be the primary psychoactive ingredient.[2] Today this substances are known as phytocannabinoids. In the early 1990s the G-protein coupled cannabinoid receptors (CB) were discovered. Two types of cannabinoid receptors exist and their name is CB1 and CB2. They are important in mechanism of THC. 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

The basic material of Cannabis products is the plant *C. sativa* L. (**Figure 1**). It 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 4–6-month. However, in modern breeding and cultivation of recreational Cannabis, the preferred way to propagate the plants is by cloning, using cuttings of the so-called mother plant. The female plants are used for this purpose, because they produce significantly higher amounts of psychoactive compounds than the male plants. 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.

Figure 1. *Illustration of Cannabis sativa L. (Köhler's Medizinal-Pflanzen, 1887, Franz Eugen Köhler).*

The female plant then produces several crowded clusters of individual flowers (flower tops); a large one at the top of the stem and several smaller ones on each branch, whereas the male flowers hang in loose clusters along a relatively leafless upright branch. The male plants finish shedding their pollen and die before the seeds in the female plants ripen, that is 4–8 weeks after being fertilized. A large female can produce over 1 kg of seed. If the seed survives, it may germinate the next spring.

According to current botanical classification, Cannabis belongs, only with *Humulus* (hops), to the small family of Cannabinaceae (also Cannabaceae or Cannabidaceae). Despite this close relationship, cannabinoids can only be found in *C. sativa*. The genus *Humulus* has been studied by scientists but in this plant there aren't cannabinoids and instead a variety of the so-called bitter acids, such as humulone, adhumulone, and cohumulone are produced. The close relationship between both plant species is clearly shown by the fact that both compounds (cannabinoids and bitter acids, respectively) are derived from similar biosynthetic pathways. Furthermore, both are excreted as a resinous mixture by glandular hairs, mainly found on female flowers.

The current systematic classification of Cannabis is:

Division Angiosperms
Class Dicotyledon
Subclass Archichlamydeae
Order Urticales
Family Cannabinaceae
Genus *Cannabis* Species *sativa* L.

Today, more than 700 different cultivars have been described and many more are thought to exist.[4]

A Short History of Cannabis

Cannabis most likely originates from Central Asia, as archeological evidence indicates that it was already cultivated in China for food and fiber 10.000 years ago. In fact, Cannabis is one of the oldest known medicinal plants and is described in almost every ancient handbook on plant medicine, most commonly in the form of a tincture or a tea. Some religions use the components of the Cannabis plant: for example, in Hindu legend, Cannabis is believed to be the favorite food of the god Shiva, because of its energizing properties. Nowadays, varieties of Cannabis can be found in all temperate and tropical zones, except in humid, tropical rain forests. The medicinal use of Cannabis was introduced in Europe only around 1840, by a young Irish doctor, William O'Shaughnessy, who served for the East India Trading Company in India, where the medicinal use of Cannabis was widespread.

At the top of its popularity, more than 28 different medicinal preparations were available with Cannabis as active ingredient, which were recommended for indications as various as menstrual cramps, asthma, cough, insomnia, support of birth labor, migraine and throat infection. However, because no tools existed for quality control, it was impossible to prepare a standardized medicine, so patients often received a dose that was either too low, having no effect, or too high, resulting in serious side effects. Moreover, Cannabis extract was not water-soluble and therefore could not be injected, moreover oral administration was found to be unreliable because of its slow and erratic absorption. Because of such drawbacks, the medicinal use of Cannabis increasingly disappeared in the beginning of the twentieth century, and in 1937 Cannabis was removed from the US pharmacopoeia, a move that was followed by most other Western countries. Isolation and structure elucidation of the first pure active substances from Cannabis has come in 1960 and so, after that, the scientists have studied for having medicines based on cannabis, who had positive effects.[4]

Chemical Constituents of Cannabis

We know 538 constituents, Cannabis is one of the chemically best-studied plants. Most of these constituents have not yet been properly characterized for biological activity and so the Cannabis plant could be called a 'neglected pharmacological treasure trove'. The most important classes are listed in **Table 1**.

Table 1 An overview of compounds identified in Cannabis

Compound class	*Compounds identified*
Terpenoids	>120
Cannabinoids	>70
Hydrocarbons	50
Sugars and related compounds	34
Nitrogenous compounds	27
Noncannabinoid phenols	25
Flavonoids	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, as shown in **Figure 2**.

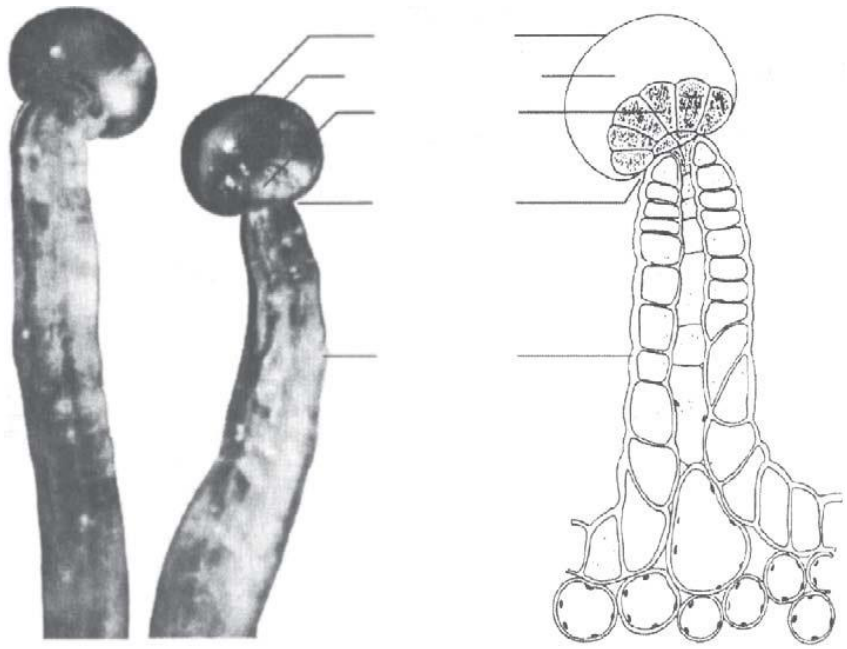


Figure 2. *Microscope photograph and drawing of a Cannabis resin gland, with secretory head cells visible underneath the transparent cannabinoid- and terpenoid- rich resin.*[4]

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. The resin gives a certain defense against insect and fungal attack and so they are useful for her protection. It has been shown that the cannabinoids, cannabigerolic acid (CBGA) and THCA, induce cell death through apoptosis for example in insect cells. It is therefore not certain how many compounds, identified from such materials, should be considered as artifacts, resulting from oxidation, and enzymatic, thermal, or other degradation. In fact, even THC itself is not produced by the metabolism of the Cannabis plant but rather is formed by thermal decarboxylation (loss of CO2) of THCA. Further degradation of THC results in the formation of cannabinol (CBN) or delta-8-tetrahydrocannabinol (Δ^8-THC). Moreover many terpenoids are known to be susceptible to degradation upon

storage or extraction. As such, the chemical composition of any given Cannabis preparation depends not only on its biosynthetic composition, but also on factors such as age, conditions of storage, and method of extraction.[4]

Cannabinoids

Cannabinoids are considered to be the main biologically active constituents of the Cannabis plant, and they can be found nowhere else in nature. This characteristic has underlined that the majority of biological activities, attributed to Cannabis, have linked to cannabinoids and more specifically to THC. The natural cannabinoids form a complex group of similar compounds of which currently 70 are known and well described. New cannabinoids, although present in very minor quantities have been discovered very recently (**Figure 3**).

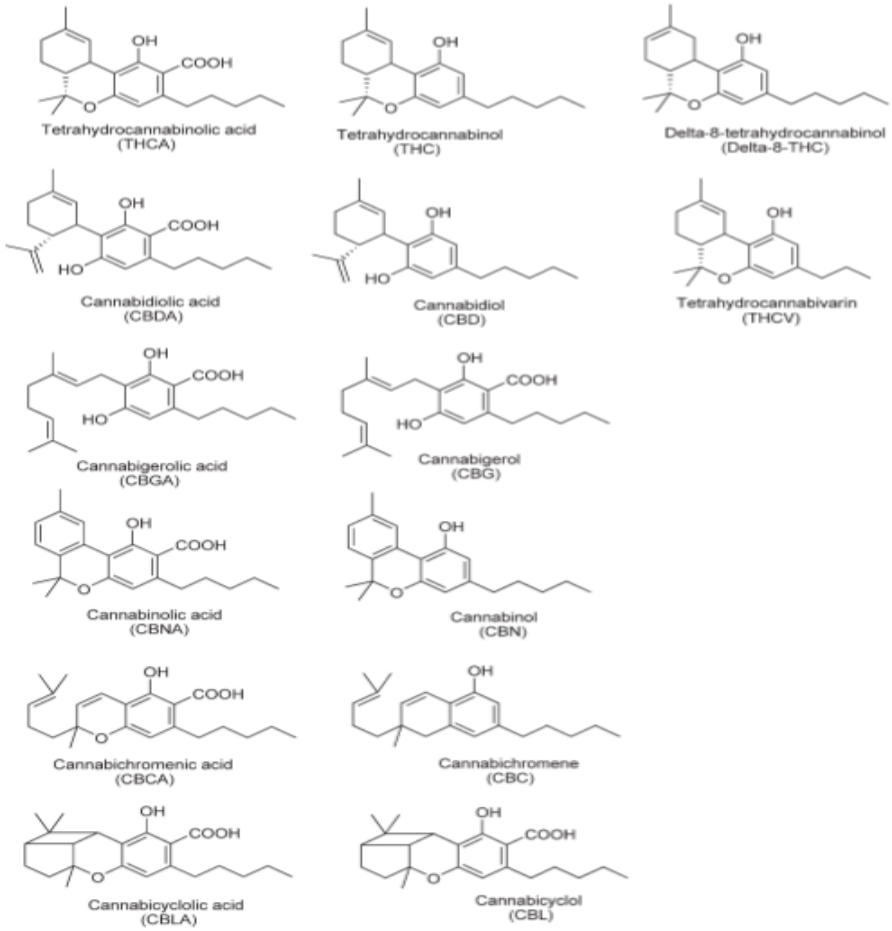


Figure 3. *Structures of the cannabinoids most commonly found in Cannabis plant materials. All cannabinoids have the (6aR,10aR)-orientation.*[4]

Until the 1980s, the term cannabinoids represented by definition the group of typical terpenophenolic C21 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 have an effect on 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. The plant-derived cannabinoids are now often termed phytocannabinoids. The first cannabinoid was isolated in 1940, and its name was CBN (C21H26O2), but it was inactive as psychoactive compounds.[4]

The most important phytocannabinoids are Δ-9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) (**Figure 4**).

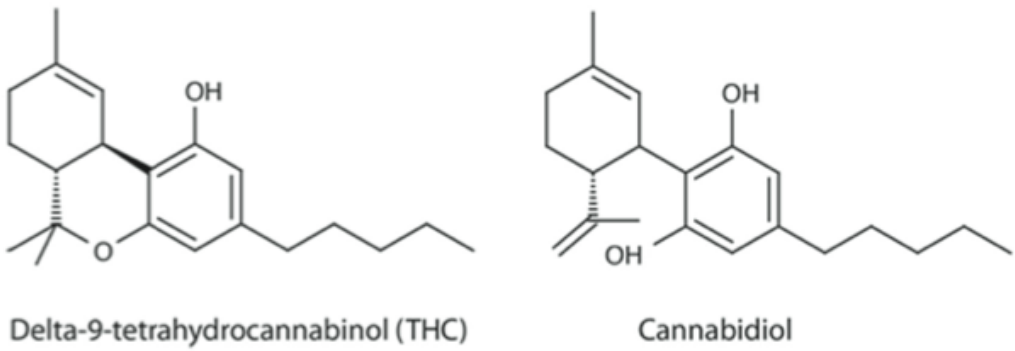


Figure 4. *The chemical structures of Delta-9-tetrahydrocannabinol (THC) and Cannabidiol.*

The scientists are inspired to Δ^9-tetrahydrocannabinol always, because it has a high affinity to CB1 and CB2 receptors and so they have projected a lot of molecules basing on the structures of this principle cannabinoid. When the scientist have begun to test Δ^9-THC and the analogous compounds, they have seen that molecules had psycothropic effects and so they have understood that the psycothropic effect of *Cannabis* was caused by Δ^9-tetrahydrocannabinol and so the similar compounds provoked the same collateral effect.

For many years it has been thought that it was opportune working for eliminating this probleme, for example modifying the structure of Δ^9-THC and various analogues.[5,6]

In the last years there has been a new discovery, the other principle compound of *Cannabis*, Cannabidiol, was studied by the scientists, because they have noted that this substance does not have the psycothropic effects of Δ^9-tetrahydrocannabinol, because it has a low affinity for cannabinoid receptors. For this reason the researchers have wanted to investigate on how this compound, when it is administered or taken, exerts a lot of healthy effects and a little of collateral effects.

Cannabidiol has anti-oxidant, anti-inflammatory and immunomodulatory effects. Moreover this substance is known to inhibit cancer cell invasion, angiogenesis and to decrease the growth of breast carcinoma and lung metastasis. This molecule activates cannabinoid receptors indirectly or rather it enhances levels of endocannabinoids, for example 2-arachidonoyglycerol in colorectal carcinoma cells and this action is chemopreventive.
Furthermore it has been discovered that cannabidiol inhibits the cellular uptake and enzymatic hydrolysis of the endogenous cannabinoid anandamide, which is involved in pain control through activation of CB1 and CB2 receptors; cannabidiol behaves as agonist of TRPV1, but we can not say whether it activates vanilloid receptors directly or indirectly, through anandamide (TRPV1 receptors are up-regulated after inflammation and nerve injury and this may contribute to inflammatory and neuropathic hyperalgesia, so the therapeutic action of cannabidiol could be due to desensitization of this receptor, similarly to the natural agonist (capsaicin)).[7,8,9]

Biosynthesis of the Cannabinoids

In all biosynthetic pathways for cannabinoids that were postulated until 1964, 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, cannabigerol (CBG) is the common precursor of cannabinoids, biosynthesized through the condensation of geranyldiphosphate (GPP) 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 today 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 group (–COOH). The first specific biosynthetic step is the condensation of GPP with OA into cannabigerol 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 THCA, CBDA, and cannabichromenic acid (CBCA), respectively. The terpenoid GPP 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 5**).[4]

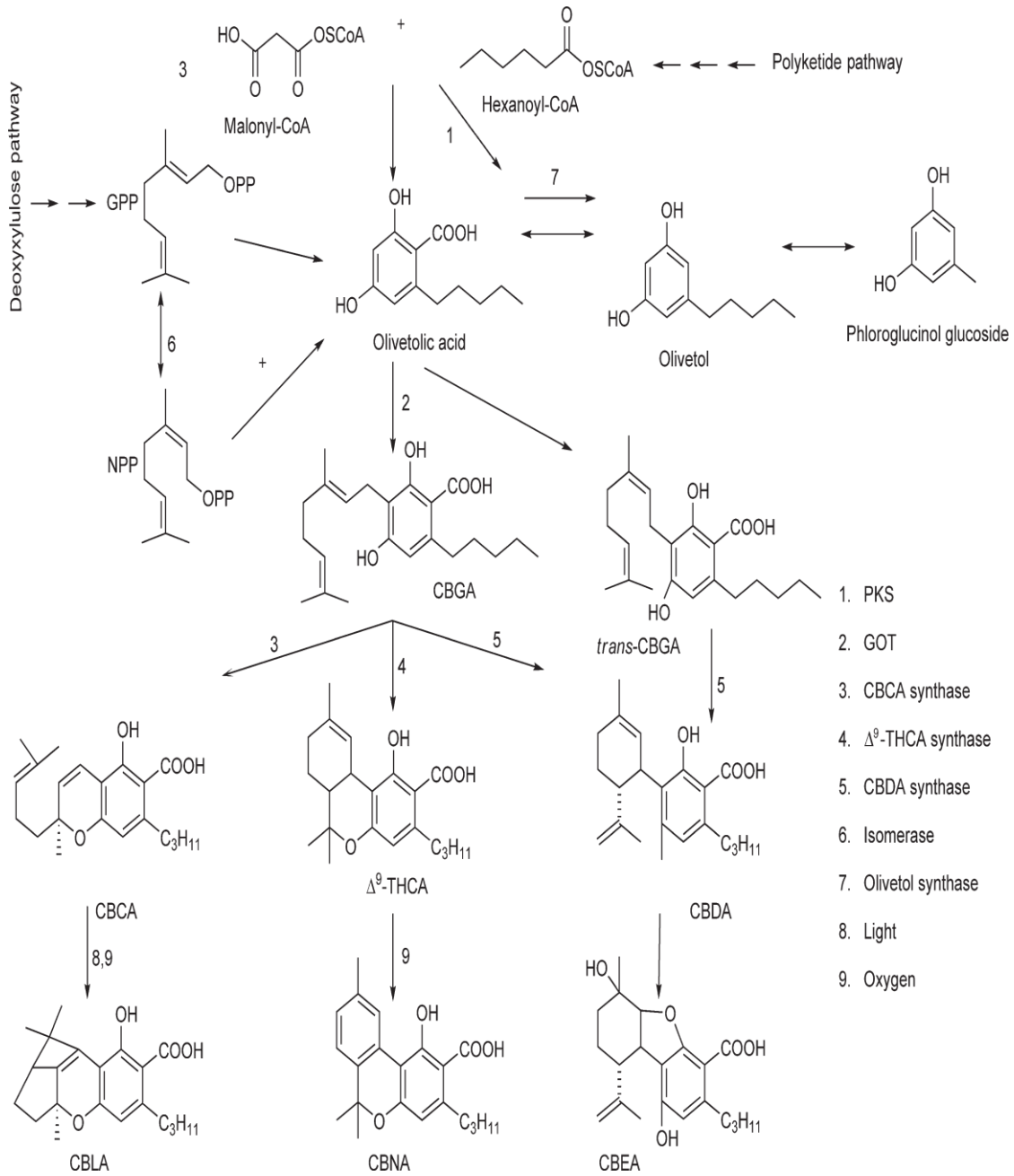


Figure 5. *General overview of the biosynthesis of cannabinoids and putative routes.*[4]

The Cannabinoid Receptors: CB1 and CB2

The cannabinoid receptors are a class of receptors under the G-protein-coupled receptor superfamily. Cannabinoid receptors have a protein structure defined by an array of seven transmembrane-spanning helices with intervening intracellular loops and a C-terminal domain that can associate with G proteins of the Gi/o family (**Figure 6)**. Their ligands are known as cannabinoids or endocannabinoids depending on whether they come from external or internal (endogenous) sources. Until the discovery of specific Cannabis receptors, the biochemical mode of action of cannabinoids was much debated. The cannabinoids have a lipophilic character and so they have a high possibility to penetrate cellular membranes by simple diffusion. Therefore, possible explanations for cannabinoid activity initially included unspecific membrane binding and it causes an increase in fluidity and permeability of neural membranes, inhibition of acetylcholine synthesis, a rise in the synthesis of catecholamines, and an interaction with the synaptosomal uptake of serotonin. However, it was established in the middle 1980s that cannabinoid activity is highly stereoselective, indicating the existence of a receptor-mediated mechanism.

The first reliable indications that cannabinoids act through receptors came when it was shown that cannabinoids can act as inhibitors of the adenylate cyclase second messenger pathway in brain tissue and neuroblastoma cell lines. This activity was dose-dependent and stereospecific. Finally, a stereospecific G-protein-coupled cannabinoid receptor was found and cloned. It was named 'cannabinoid-binding receptor type 1' or CB1R. The CB1 receptor is most clearly localized in the central nervous system (CNS), therefore it is often called the 'central receptor', but it is also found in certain peripheral organs and tissues, such as lungs, liver, and kidneys. CB1 receptors are thought to be the most widely expressed G-protein-coupled receptors in the brain. Activation leads to the inhibition of adenylate cyclase activity. The CB1 receptor also modulates ion channels, inducing, for example, the inhibition of N- and P/Q- type voltage-sensitive $Ca2^{+}$ channels and the activation of G-protein inwardly rectifying K^{+}

channels. The cannabinoid receptors have also shown to modulate several signaling pathways that are more directly involved in the control of cell proliferation and survival, including extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/Akt, focal adhesion kinase (FAK), and the ceramide pathway. Subsequently, a second cannabinoid receptor (CB2R) was found with a possible role in immunological processes. The CB2 receptor was first described as a peripheral G-protein-coupled receptor (GPCR), mainly localized in the immune system, therefore it is often called the 'peripheral receptor'. However, nowadays it is well know that CB2R expression is hight in neurons of the brain too. It is important to underline that CB2R is primarily expressed by immune tissues such as leukocytes, spleen, and tonsils, and it shows a different selectivity than the centrally acting CB1R. So far, the physiological roles of CB2 receptors are proving more difficult to establish, but at least one seems to be the modulation of cytokine release. Recently, it has been recognized that CB2R may play a functionally relevant role in the CNS, mediated through microglial cells. It's important to say the protein sequences of CB1R and CB2R show only about 45% homology.[4] However, not all of the effects of cannabinoids can be explained by receptor-mediated effects, and it is believed that at least some effects are nonspecific and caused through membrane perturbation.[10]

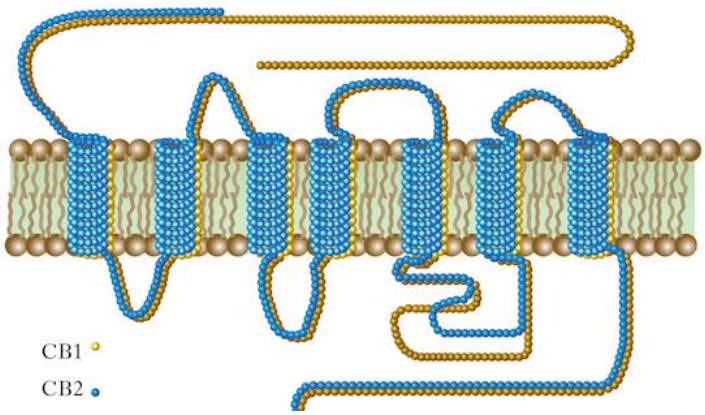


Figure 6. *CB1 and CB2 receptors structures (Dizionario di Medicina, 2010, Treccani).*

The Endocannabinoid System

Based on the observation that all natural cannabinoids are highly lipid-soluble, an attempt was made to isolate endogenous ligands for the cannabinoid receptors from fatty tissues of animals. Finally, a single compound could be isolated from the brain tissue of pigs and it has a high affinity for the CB1 receptor. It was chemically identified as arachidonic acid ethanolamide, and named anandamide (AEA). A few years later, a related compound was isolated from canine gut with an affinity for both cannabinoid receptors: 2-arachidonylglycerol (2-AG). Structures of these two compounds are shown in **Figure 7**. In recent years, a variety of compounds with endocannabinoid activity have been isolated or synthesized, interestingly, all having an eicosanoid-related structure.

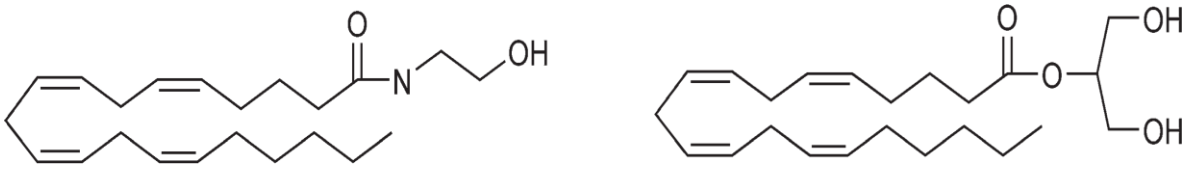


Figure 7. *Structures of the two major endocannabinoids.*[4]

Discovery of cannabinoid receptors and their endogenous ligands allowed to the identification of a ubiquitous neuromodulatory system called endocannabinoid system.

It consists of cannabinoid receptors, endogenous cannabinoids, and enzymes responsible for their production, transport, and degradation. The endocannabinoid

system can be found even in very primitive organisms, indicating it has a fundamental role in basic physiology. There are currently two main families of endocannabinoids that have been extensively characterized. The first are amides of arachidonic acid and ethanolamide; the typical example of this family is anandamide. The second family includes glycerol esters related to 2-AG. The biosynthetic pathways for both families of endocannabinoids are complex. There are several pathways known for the synthesis and degradation of endocannabinoids. Basically, endocannabinoids exist intracellularly as precursors in the plasma membrane of neurons as part of certain phospholipids. They are produced on demand 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 causes cell depolarization or the mobilization of intracellular calcium stores subsequent to the stimulation of protein-coupled receptors from the Gq/G11 family (**Figure 8**).

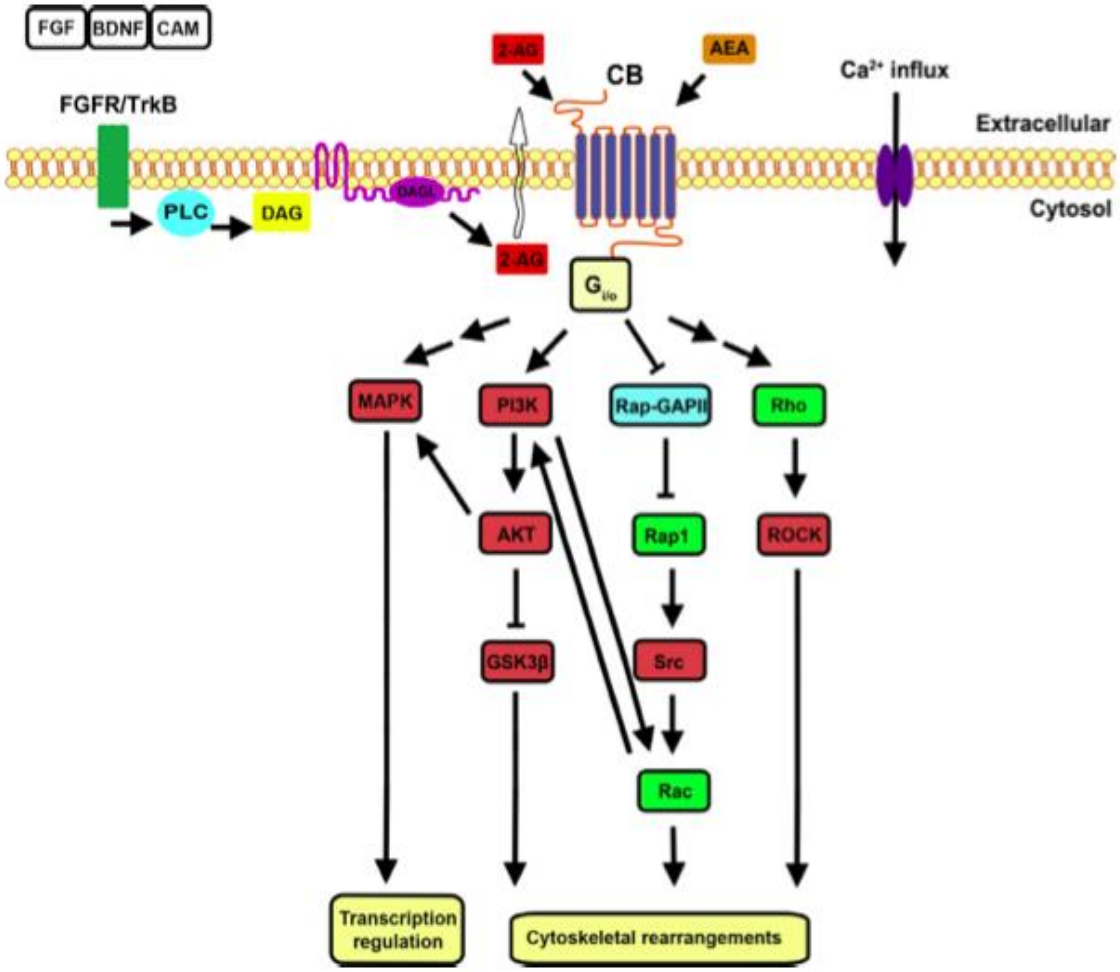


Figure 8. *An overview of the eCB signalling system in neuronal migration. DAGL-dependent local synthesis of 2-AG, the main eCB in the brain, can occur downstream of tyrosine kinase receptors (such as FGF and TrkB). Cell adhesion molecules (N-cadherin/NCAM) can also cross-talk with the FGF receptor. Following release of 2-AG (or after binding of other eCBs like anandamide), CB receptor activation can trigger stimulation of PI3K and MAPK pathways and activation of small GTPases such as Rap1, Rac and Rho, potentially regulating migration by affecting translation, adhesion and cytoskeletal rearrangement (Zhou Y. et al., The International Journal of Biochemistry & Cell Biology, 47, (2014), pp.104-108).*

Accordingly, the enzymes catalyzing anandamide and 2-AG are calcium-sensitive. After formation, endocannabinoids are transported, across the cell membrane for interaction with their extracellular binding sites, on cannabinoid receptor (**Figure 9**).

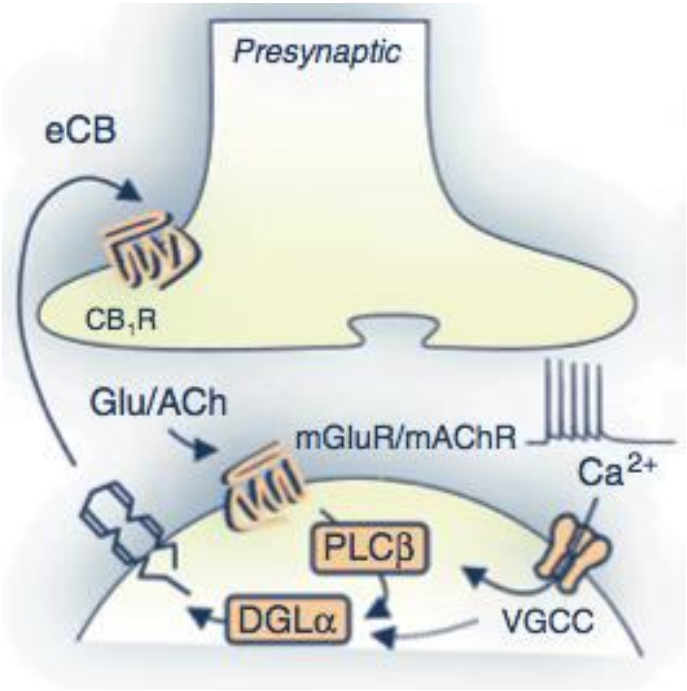


Figure 9. *Postsynaptic activity (e.g. Ca2+ influx through voltage-gated Ca2+ channels, VGCCs) along with metabotropic receptor activation (e.g. mGluR and mAChR), (Younts T.J., Castillo P. E., Current Opinion in Neurobiology, 26, (2014), pp.42-50).*

Endocannabinoids differ from other neurotransmitters such as acetylcholine, gamma-aminobutyric acid (GABA) and dopamine since they most commonly travel backward against the usual synaptic transmitter flow. In fact, endocannabinoid signaling appears to occur via a retrograde mechanism: stimulation of the postsynaptic neuron triggers the biosynthesis of endocannabinoids, which are rekeased and transported to the presynaptic cell, where the target receptors are densely concentrated. The endocannabinoids exert a homeostatic function. Because of their chemical (non water-soluble) nature, they cannot travel unaided for long distances in the aqueous medium

surrounding the cells from which they are released. Therefore, endocannabinoids do not typically function like hormones, they act as local (autocrine or paracrine) mediators. Activation of the cannabinoid receptors temporarily reduces the amount of conventional neurotransmitter released, since CBRs activation of G proteins initiates a signaling cascade that the inhibits the activity of voltage-gated calcium channels, thus reducing entry of Ca^{2+}, and thereby forbidding the exocytosis of secretory vesicles. The ultimate outcome of cannabinoid signaling depends on the nature of the participating cells: endocannabinoid signaling will be inhibitory, if CB receptors are activated on glutamatergic neurons, whereas the net result will be disinhibitory (or excitatory) if CBRs activation takes a place on GABAergic neurons.

When the local action of endocannabinoids has not neuron like target, but glial cells and microglia, stimulation of CB1R and primarily CB2R regulates many important functions, such as migration and activation, the release of pro- and anti-inflammatory cytokines and the ability of adjusting the neurotransmission by the release of neurotransmitters or seizure.

Degradation is an important mechanism to regulate endocannabinoid activity, as the duration of endocannabinoid effect is dependent on the localization of the degrading enzymes. The degradation system involves reuptake into the presynaptic cell, followed by rapid hydrolysis of the amide or ester bonds. We do not know how endocannabinoids move from the extracellular space to the interior of a cell, but we have understood that they utilise specific proteins for faciliting the membrane transport. It has been discovered that 2-AG exhibits higher selectivity and efficacy for CB1 and CB2 receptors than anandamide, which also interacts with noncannabinoid receptor targets. Therefore the levels of the two compounds are regulated in different ways. However, the main enzyme that inactivates both anandamide and 2-AG (and others) by hydrolysis is fatty acid amide hydrolase (FAAH). 2-AG is also inactivated by monoacylglycerol lipase (MAGL) (**Figure 10**).[4]

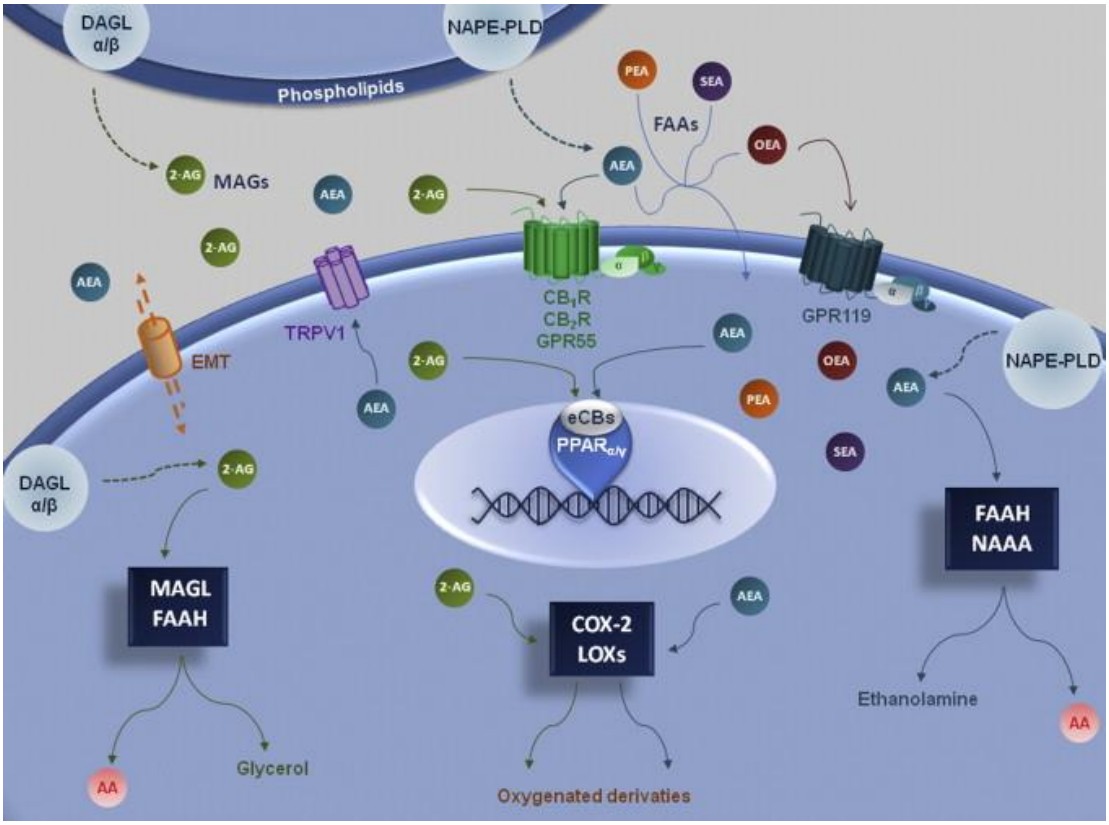


Figure 10. *Endocannabinoid synthesis and degradation pathways (D'Addario C. et al., Neuroscience & Biobehavioral Reviews, 47, (2014), pp. 203-224.*

From Endocannabinoid System to "Endocannabinoidome"

The endocannabinoid (eCB) system consists of receptors, endogenous ligands, and ligand metabolic enzymes. Metaphorically the eCB system represents a microcosm of psychoneuroimmunology or mind-body medicine. Cannabinoid receptor 1 (CB1R) is the most abundant G protein-coupled receptor expressed in the brain, but it is also expressed in non-neuronal cells, such as adipocytes and hepatocytes, and in musculoskeletal tissues. Cannabinoid receptor 2 (CB2R) is principally associated with cells governing immune function, although it may also be expressed in the central nervous system.
The eCB ligands are anandamide (AEA) and 2-arachidonoylglycerol (2-AG). AEA is biosynthesized by N-acyl-phosphatidylethanolamine phospholipase D (NAPE-PLD) and 2-AG by two isoforms of diacylglycerol lipase, DAGLα and DAGLβ. After that these compounds have conducted their function, they are catabolized, AEA by FAAH1 and 2-AG by MAGL.

In the last years this system has undergone to an expansion, because there has been the discovery of secondary receptors, ligands, and ligand metabolic enzymes. For example, AEA, 2-AG, N-arachidonoyl glycine (NAGly) and the phytocannabinoids Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) may also serve, to different extents, as ligands at GPR55, GPR18, GPR119, and several transient receptor potential ion channels (TRPV1, TRPV2, TRPA1, TRPM8).

Moreover one of the most meaningful discoveries has been that the effects of AEA and 2-AG can be enhanced by ‘‘entourage compounds’’ that inhibit their hydrolysis via substrate competition, and thereby prolong their action. Entourage compounds include N-palmitylethanolamide (PEA), N-oleoylethanolamide (SEA), and cis-9-octadecenoamide (OEA, oleamide).[11]

Particularly with regard to anandamide, the researchers have studied its uptake and the involved subjects in this action. Recent studies have identified

intracellular transport proteins (fatty acid binding proteins 5 and 7, heat shock protein 70, albumin, and fatty acid amide hydrolase-like AEA transporter protein) that shuttle AEA from the plasma membrane to its metabolic enzymes. The anandamide needs of a carrier for crossing the plasma membrane, but it is unclear why a lipophilic molecule such as AEA would need 'help'. It is thought that an FAAH-driven AEA uptake can be generalised. A lot of intracellular AEA carrier proteins have been proposed and given that AEA carriers must be ubiquitous and able to bind lipophilic compounds, the scientists have indentified fatty acid binding proteins (FABPs).

Most recently studies have underlined the identification of a variant of FAAH that was catalytically inactive, but which bound AEA, it was FLAT (FAAH-like anandamide transporter). This carrier lacks the membrane-anchoring domain of the FAAH dimer. FLAT is an important protein, that transports anandamide in cells, where it will be destroyed, but this protein does not degrade it. It has been proved taht in mouse brain neurons, genetic deletion of FAAH (which eliminates both FAAH and FLAT) reduces but does not eliminate AEA uptake (**Figure 11**).

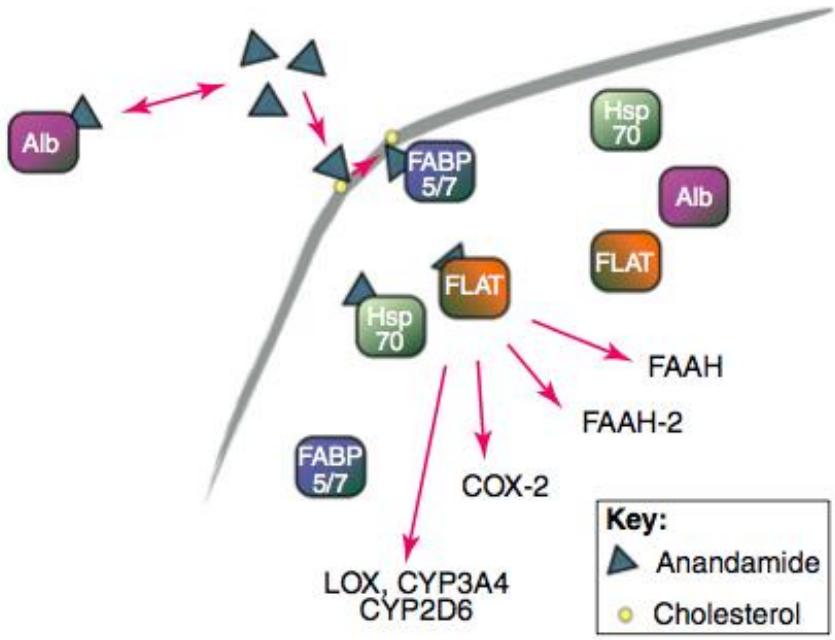


Figure 11. *Conceptualised uptake and metabolism of anandamide (AEA) in a model, which does not invoke the presence of a plasma-membrane transporter protein.*[12]

Furthermore a recent study has spoken about the transient receptor potential vanilloid 1 (TRPV1), a receptor, which behaves like a biosensor to measure the intracellular accumulation of AEA, but about this the knowledges are still little.

The ongoing studies are based on three areas:

1) Uptake versus endocytosis of AEA, because there is evidence that AEA can be internalised by endocytotic processes, although some of the compounds used to disrupt endocytosis are far from selective. Further research is necessary to determine whether cells use both endocytotic and transmembrane mechanisms of AEA uptake or other processes;

2) Uptake of 2-AG, which has been identified as the endocannabinoid primarily involved in many retrograde signalling processes. In this case, both the target CB1 receptors and the main metabolic enzyme for 2-AG are located presynaptically and a plasma-membrane transporter would be expected to compete with the receptor for 2-AG. This may be an elegant way of regulating the degree of retrograde signalling, or alternatively an argument against a membrane transporter.

3) Balance of intracellular carriers and metabolic enzymes in pathological conditions: ischaemia, for example, changes hippocampal expression of FABP5, FABP7 and Hsp70. Cytokines can affect FAAH expression, so it is not inconceivable that FLAT expression might also be affected. Changes in these characteristics modify the accumulation and metabolism of AEA and in this way novel possibilities for therapeutic intervention is created.[12]

However, endocannabinoids and AEA in particular, may interact with other targets besides to “classic targets” and produce other positive effects, for example in case of TRPV1 activation, pro-nociceptive effects.

Moreover, the existence of multiple endocannabinoid-degradation pathways allows the realization of new inhibitors and in this way an enhancement of

endocannabinoid levels is caused, besides this the presence of other receptors, definied “orphan receptor”, involved in endocannabinoid system, gives to the researcher a new possibility of using them like new targets. Naturally, as above described, the new pharmacologic opportunities, that “Endocannabinoidome” offers, are a lot. Consequetly, considering that a multi-target approach has been reached, a polypharmacology treatment has been obtained and so the effectiveness of therapy has been increased.[13]

Role of CB1R and CB2R in Neuroprotection

The CB1 and CB2 receptors have widely been associated with neuroprotection. The most extensively studied mechanism of neuroprotection includes the antinflammatory effects of the CB2 receptor. 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. In like manner, cannabinoid receptor ligands are often reported to be antioxidative.[14]

It is well known that CB1R and CB2R activities are strictly linked to their respectively localization. In fact, most of physiological functions associated with the CB2 receptor deal with different types of immunological effects given the predominance of this receptor type in immune tissues. However, its recent description in certain brain regions allows us to relate different neurobiological processes, for example control of pain, brain reward, emotion and others to CB2 receptor activity.[15] Interestingly is the implication of the CB2 receptor in processes related to the control of proliferation[16], differentiation[17] and survival[18] of neural cells. The CB2 receptor plays an important role in this process because it provides cytoprotection of healthy neural cells or apoptosis of tumoral cells. The former property, more than the lattest, is related to CB2 receptor capability to arrest/delay brain damage in different neurodegenerative disorders, particularly in those that exhibit an important local inflammatory component associated with brain injury. Also CB1 receptor is involved in neuroprotection mechanism since it is associated with inhibition of glutamate release, decrease of cytosolic free Ca2+ concentration and vasodilation, effects that are overall capable to increase neuronal survival.[19] This potential, together with other cannabinoid receptor-independent properties allows cannabinoids to protect neurons from death (cerebral ischemia and trauma) and from chronic neurodegenerative disorders (Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS)).

Role of CB1R and CB2R in Neuropathogenesis

CB1 and CB2 receptors have been implicated in a number of neuropathogenic processes. However, the cannabinoid receptor type correlated to an inflammatory response within the CNS appears to be CB2R. Microglia apparently act as the major expressers of this receptor during early stages of neuroinflammation. In the CNS resident macrophages migrate and proliferate during and after injury and inflammation, phagocytose and process antigens, and once activated produce pro-inflammatory 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. Paradoxically, microglia not only play a role in host defense and tissue repair in the CNS, but also have been implicated as contributing to nervous system disorders such as Mutiple Sclerosis (MS). This deleterious role of microglia is exacerbated by inflammatory factors that are elicited from microglia and astrocytes contribute to breakdown of the Blood Brain Barrier (BBB) and play a critical role in the influx into the CNS of immunocytes from peripheral non-neuronal sites that also express CB2R. *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 pro-inflammatory states. There are also evidences that expression of CB2 receptor is up-regulated *in vitro* by microglia and other immune cells in response to immune modulators and *in vivo* during states of chronic neuroinflammation. Thus, using CB2-selective agonists it may be possible to target specific cell types such as microglia that are associated with inflammatory processes in the brain.[20]

Alzheimer's disease (AD)

AD is the most common 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 is caused by effects, that multiple neurotransmitter systems induces, 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. This characteristic 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 (**Figure 12**). 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. 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, CB2R and FAAH have been reported to be selectively over-expressed in neuritic plaque associated glia in AD brains.

Immunohistochemical staining of hippocampus and entorhinal cortex sections demonstrated that both FAAH and CB2R were abundantly and selectively expressed in neuritic plaque associated astrocytes and microglia. Intracerebroventricular administration of the 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 WIN55212-2, and two other CBR agonists, HU-210 and JWH-133 resulted in blockage of their activation by Aβ peptide and abrogated neurotoxicity mediated by these cells. These results indicate that WIN-55212-2 exerted anti-inflammatory properties in a model of chronic brain inflammation produced by infusion of bacterial lipopolysaccharide (LPS) into the fourth ventricle of young rats. It was suggested that WIN-55212-2 exerted an indirect effect on activation of microglia and impairment of memory. On the other hand, it has been reported that stimulation of CB2R results in suppression of inflammatory mediators such as NO, cytokines, and chemokines that play a role in microglial cell-associated neuronal damage.[20]

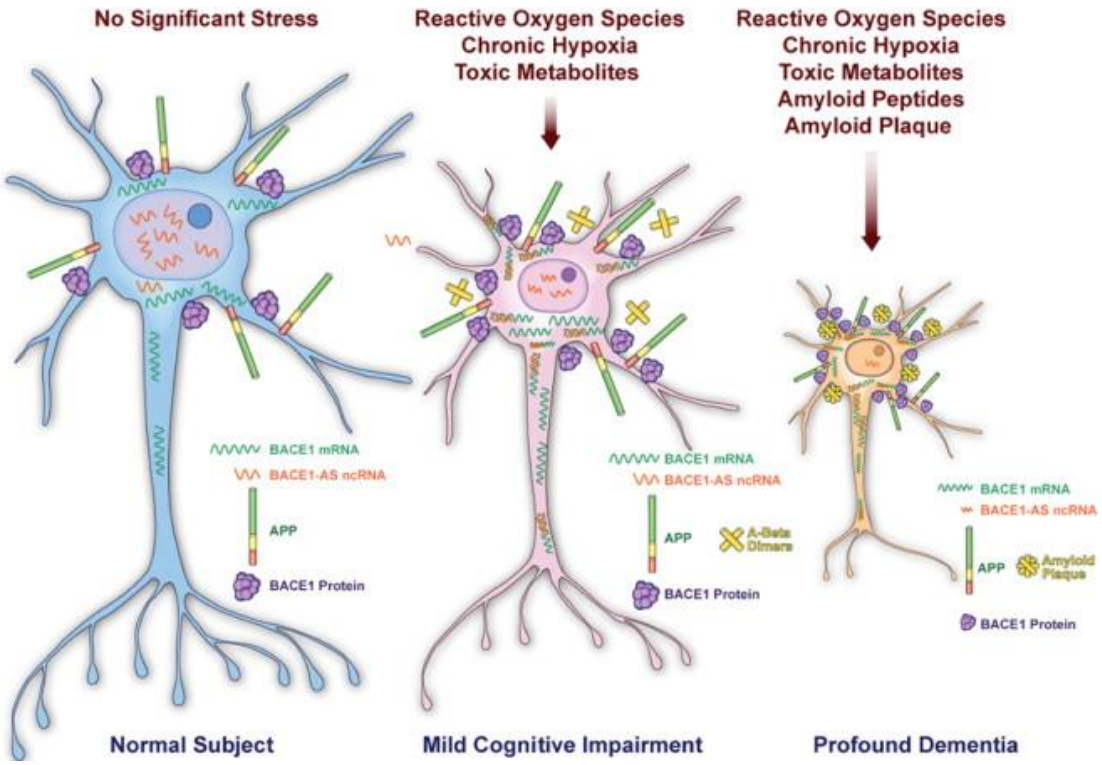


Figure 12. *Immagine of neuron in normal subject, mild cognitive impairment and profound dementia (Faghihi M.A. et al., Neuroscience Letters, 466, (2009), pp.81-88).*

Multiple Sclerosis (MS)

MS is also known as “disseminated sclerosis” or “encephalomyelitis disseminate”. It is a chronic, inflammatory demyelinating disease of the human CNS that primarily affects adults. This pathology causes muscle weakness, abnormal muscle spasms, changes in sensation, difficulty with 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. These 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.

Thanks to several experiments, it is well understod that demyelination in the CNS is due to migration of activated myelin-specific T lymphocytes across the tight endothelial junctions that comprise the BBB and from the elaboration of chemokines and cytokines by these T lymphocytes. The resultant influx of peripheral mononuclear phagocytes into the CNS leads to progressive hind- limb paralysis.[21]

Most studies, with aim for discovering the effects of cannabinoids on MS have involved the use of the mouse EAE model (mouse with Experimental Autoimmune Encephalomyelitis) and it is caused by Theiler’s murine encephalomyelitis virus, that induces demyelinating disease (TMEV-IDD). Mouse deficient in CB1R have been shown to tolerate inflammatory and excitotoxic insults poorly and to develop substantial neurodegeneration following immune attack.[22] Afterwards with these

observations, THC has been reported to markedly inhibit neurodegeneration in the EAE model and to reduce the associated induced elevated level of glutamate in cerebrospinal fluid. Glutamate, the major excitatory neurotransmitter in the cerebral cortex, has been implicated in neurodegenerative disease, because it is present at high levels. About this, the scientists have observed that the use of the CB1R antagonist (SR141716A) caused the inhibition of glutamate release and so they have understood that the granting of the neurotransmitter was activated by CB1 receptor. Further support for a role of the CB1R in moderating MS in experimental animals has been obtained from studies using CB1R knockout mice. These animals have shown many damages thus all the data suggested that signaling through CB1R conferred neuroprotection during EAE. The CB1 receptor exhibited down-regulatory responses that were circumscribed to motor-related regions that, generally, were more marked during the acute and chronic phases of disease. It was suggested that these observations explained the efficacy of cannabinoid agonists in improving motor symptoms such as spasticity, tremor, and ataxia that are typical of MS in humans and in animal models. Control of spasticity in the MS model as mediated by CB1R and not CB2R also has been suggested through studies in which spasticity was induced in wild type and CB1R-deficient mice following the development of relapsing EAE. CB2-selective agonists such as RWJ400065 did not inhibit spasticity. The anti-spastic activity of RWJ400065 and the therapeutic effect of the non-selective CB1R/CB2R agonists WIN55212-2 and CP55940 were lost in spastic, CB1R-deficient mice. It shows that it is not sufficient the presence of CB2R, but it is strictly necessary that of CB1R.

After it has been seen that EAE rats also have been reported to exhibit changes in endocannabinoid levels. Levels of anandamide and 2-AG were decreased in motor related regions such as the striatum and midbrain as well as in other brain regions.

The role of cannabinoids in neuroprotection in mouse and rat EAE models has been confirmed using the TMEV-IDD model. Using this animal model, treatment with WIN 55212-2, ACEA (a selective CB1R agonist) and JWH-015 (a selective CB2R agonist) during established disease resulted in ablation of neurological deficits. These cannabinoids reduced microglial activation, abrogated Major Histocompatibility Complex (MHC) class II antigen expression, and decreased the number of CD4+ infiltrating T cells into the spinal cord. WIN55212 causes an improvement in the progression of symptoms of clinical disease in mice with pre-existing TMEV-IDD. WIN 55212-2 also has been found to attenuate the increase in leukocyte rolling and endothelial adhesion in the brain that is associated with EAE. Subsequently, using CB1R (SR 141716A) and CB2R (SR144528) antagonists it was demonstrated that the cannabinoid-mediated inhibitory effects on leukocyte/endothelial interactions were through CB2R. Another important discovery was that WIN 55212-2 has been demonstrated to increase cyclooxygenase-2 (COX-2) expression and prostaglandin-E2 (PGE2) release in endothelial cells. Treatment with WIN 55212-2 resulted in up-regulation of COX-2 protein and PGE2 release that was attributed to a mechanism independent of activation of CB1R or CB2R.

More recent studies indicate that neuroprotective effects of cannabinoids can be exerted through activation of both CB1R and CB2R.

The role of ‘indirect’ agonists, compounds able to reinforce physiological endocannabinoid transmission and devoid of psychotropic effects has been examined in ameliorating neurological deficits due to MS. Using the TMEV-IDD model, it was demonstrated that treatment with the selective anandamide uptake inhibitor UCM707 during established disease resulted in significant improvement in motor function. UCM707 was able to reduce microglial activation, diminish MHC class II antigen expression, decrease cellular infiltrates in the spinal cord, and decrease the production of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6. It has been reported also

that the endocannabinoid system is highly activated during CNS inflammation and that anandamide protects neurons from inflammatory damage through a CB1R/CB2R-mediated rapid induction of microglial mitogen-activated protein kinase phosphatase-1 (mkp-1) that is associated with histone H3 phoshorylation of the mkp-1 gene sequence. It was suggested that anandamide induced a rapid mkp-1 switch off of MAPK signal transduction that was activated by stimulation of pattern recognition receptors. It was proposed that the release of anandamide served as a mode of neuroimmune communication that could control and limit the immune response after primary CNS injury (**Figure 13**).[20]

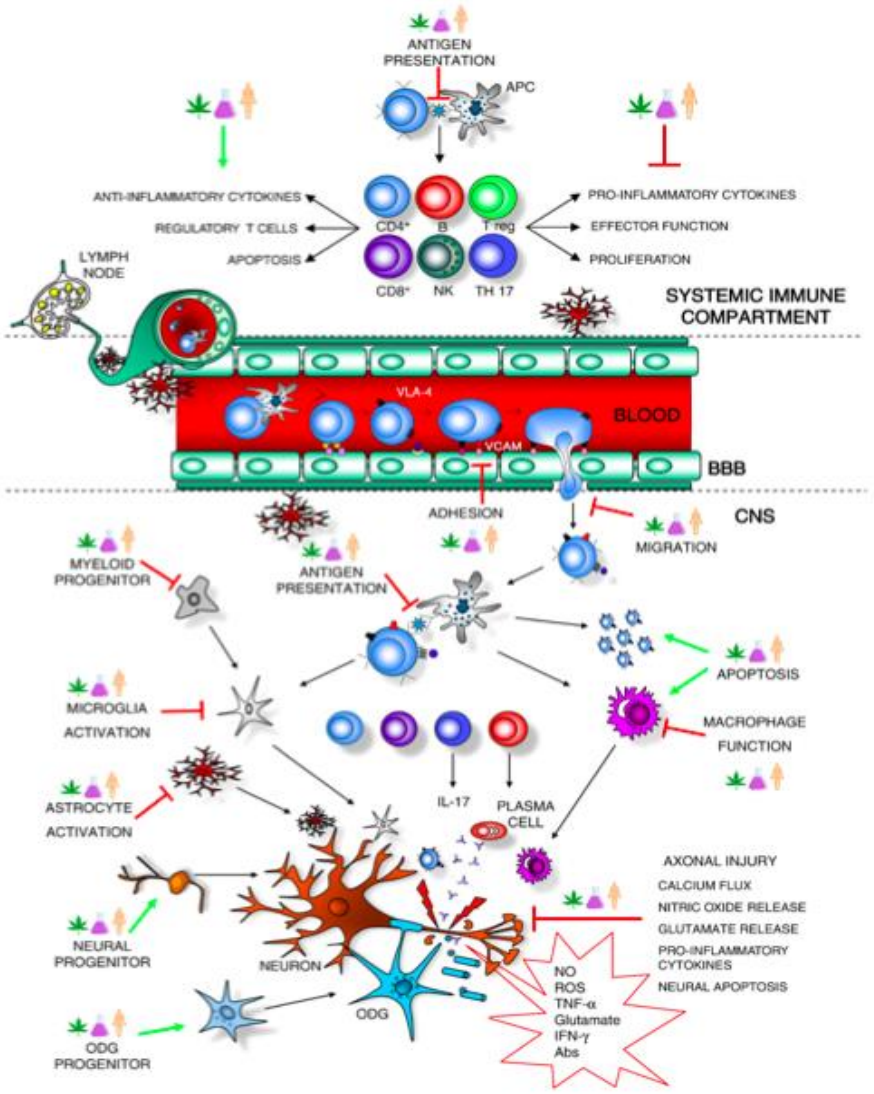


Figure 13. *The complex role of the cannabinoid system and possible therapeutic targets in MS-induced inflammation. Cannabinoids may combat inflammation by modulating cytokines, inflammatory mediators, T cell proliferation and effector function, B cell antigen presentation and activation of microglia 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.*[14]

Amyotrophic Lateral Sclerosis (ASL)

ALS is a chronic neuromuscular disease that is characterized pathologically by progressive degeneration of cortical motor neurons (upper motor neurons) and clinically by muscle wasting, weakness, and spasticity that progresses to complete paralysis. 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) by products of normal cellular metabolism. One mode involved in ALS pathology is neuroinflammmation, that is mediated by pro-inflammatory cytokines, prostaglandins, and nitric oxide (NO). More recently in the experiment with mice, it has been shown that the CB2 agonist AM-1241 has been reported as contributing to prolonged survival of SOD1 mutant mice. In addition, treatment with non-selective cannabinoid partial agonists prior to, or upon, the appearance of symptoms of ALS was shown to delay disease progression and promote prolonged survival of mice.

It has been demonstrated also that levels of endocannabinoids are affected in experimental ALS. The amount of anandamide and 2-AG, endocannabinoids that have been implicated in playing a neuroprotective role, increased in the spinal cord of SOD1 transgenic mice. Anandamide and 2-AG not only are upregulated with disease progression, but also postsymptomatic treatment with WIN55212 resulted in a delay in disease progression.[20]

Role of CB1R and CB2R in Cancer

Cannabinoids have been successfully used in the treatment of some of the side effects, such as nausea and vomiting, weight loss, lack of appetite and pain that often accompany cancer. In fact, Marinol and Cesamet (oral capsules of nabilone – a synthetic THC analogue) can be prescribed to prevent nausea and vomiting elicited by standard chemotherapeutic regimes. Although cannabinoids are used in the palliative treatment of cancer, for example Sativex is approved in Canada for the treatment of cancer-associated pain.

Unfortunately they are not yet used as a treatment for tumor progression it-self. However, aside from these palliative actions, recent preclinical evidence suggests that cancer patients might benefit from cannabinoids in an additional manner. It has been demonstrated that administration of Δ^9-THC, Δ^8-THC and cannabinol inhibited the growth of Lewis lung adenocarcinoma cell growth in vitro and in vivo after oral administration to mouse. Since then, cannabinoids have been shown to have anti-proliferative, anti-metastatic, anti-angiogenic and pro-apoptotic effects in various cancer types (lung, glioma, thyroid, lymphoma, skin, pancreas, uterus, breast and prostate carcinoma) using both in vitro and in vivo models.[23,24] Recently, more evidences have been obtained that suggest that phyto-, endo- and syn- thetic cannabinoids could be useful in the treatment of cancer due to their ability to regulate cellular signaling pathways critical for cell growth and survival.[25,26]

Role of CB1R and CB2R in 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. This burden negatively affects their life quality, functional status and life expectancy. Cancer pain is often treated with opioid 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. Cannabinoids are excellent analgesics in acute pain and chronic pain. Cannabinoids modulate nociceptive processing, because they work at the level of central and peripheral mechanisms. The majority of these effects are mediated by CB1 receptors located in both areas, but although CB2 receptors were detected in the nervous system in much lower levels than CB1 receptors. Recent experiments have demonstred that CB2R-selective ligands are more active in animal models with hyperalgesia.

The analgesic effect of cannabinoids is attributed in particular to activation of these receptors in the brain, the spinal cord and nerve terminals.

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.[27,28]

Cannabinoid Receptor Ligands

Cannabinoid receptor agonists and antagonists were reviewed in several studies (**Figure 14**). [29,30]

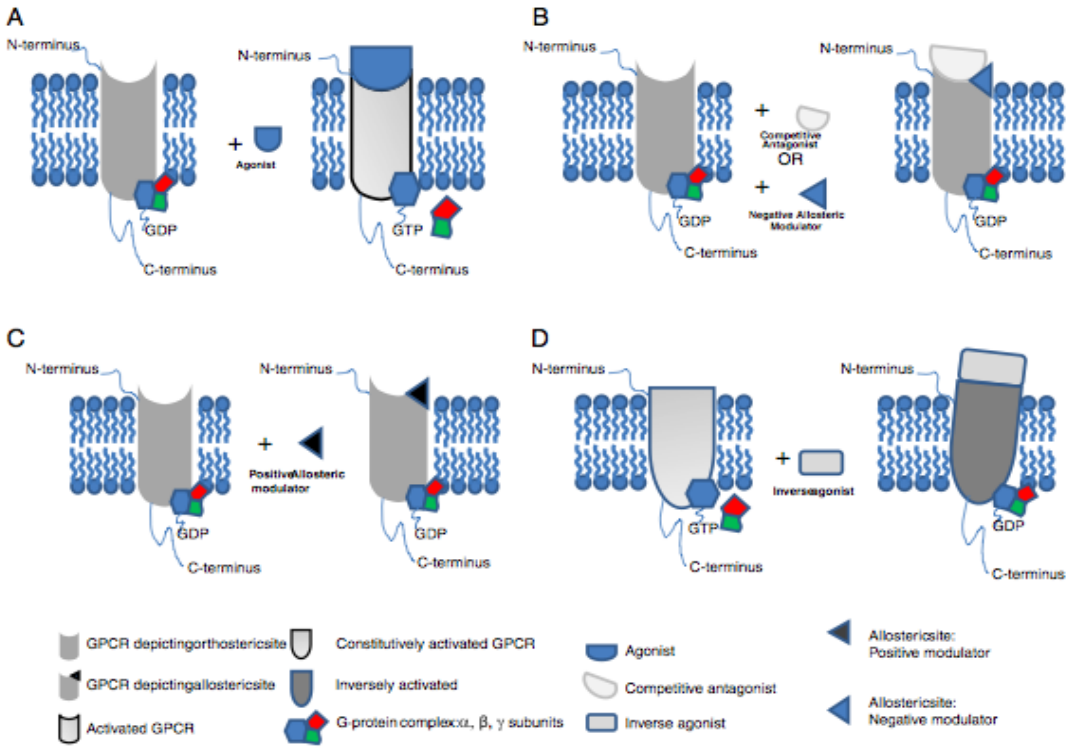


Figure 14. *Schematic of G protein coupled receptor (GPCR).*
Panel A: illustration of inactive receptor (left) and agonist-activated receptor (right). In the inactive state, the G protein–GDP bound subunit complex is bound to the receptor protein (left); whereas upon binding of agonist at the orthosteric site, the receptor is activated and the G protein subunits βγ dissociate from the GTP bound α subunit (right). Panel B illustrates binding of either competitive antagonist or negative allosteric modulator at orthosteric and allosteric sites respec- tively. Note that the receptor is not activated by either of these ligands. Panel C: binding of a positive allosteric modulator, at an allosteric site. Note that this ligand does not activate the receptor in and of itself. Panel D: constitutive activity is demonstrated on the left. The receptor is in an activated state, bound to the α subunit of the GDP–G protein complex, in the absence of agonist. Presumably in this state the receptor conformation is different from the agonist-receptor conformation, indicated by a difference in receptor shape and shad- ing. Binding of an inverse agonist, at the orthosteric site of a constitutively active receptor causes a "deactivation" of constitutive activity, a presumed conformational change, and recoupling of the G-protein subunits with the receptor. Note that the position of the G protein complex is shown bound to the receptor in a slightly different location to illustrate a different G protein-receptor conformational state, accommodating decreased basal activity consequent to inverse agonist binding.[31]

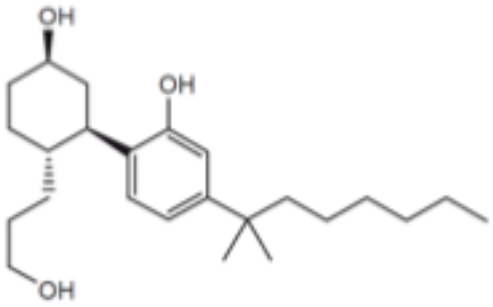
Agonists

A lot of potent agonists have been discovered and they can be subdivided into four groups according to their chemical structures. The first group (classical cannabinoids) is represented by dibenzopyran derivatives that are both natural constituents of cannabis (Δ^9-THC and Δ^8-THC) and their synthetic analogues (HU 210). The first generation of classical cannabinoids lacked CB1R/CB2R selectivity but they were developed by making relatively minor changes to the THC molecule (CB2R-selective agonists: JWH-133 and HU-308).[32,33,34] The second group (non-classical cannabinoids) are the bicylic and tricyclic analogues of Δ^9-THC lacking the pyran ring.[35,36] This group includes the main cannabinoid agonist, CP55940, which binds to both CB1 and CB2 receptors with similar affinity and displays high activity in vivo. It is 10 to 50 times more potent than Δ^9-THC in the mouse model.[35] CP55940 behaves as a full agonist for both receptor types. The third group of cannabimimetic compounds contains the aminoalkylindoles. In these series, the most important is WIN 55212-2, which displays high affinity for both CB receptors, albeit with moderate selectivity in favour of the CB2 receptors. Some of these aminoalkylindoles have been found to display significant selectivity for the CB2R (JWH-015).[37,38] The prototype of the fourth eicosanoid group, which involves arachidonic acid derivatives, is anandamide, the first endogenous cannabinoid isolated from mammalian brain.[39]

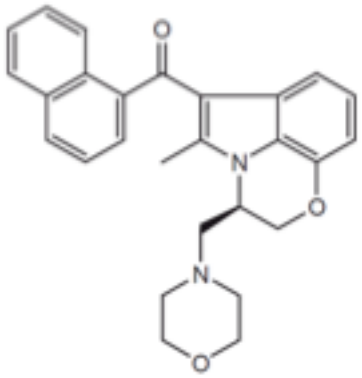
Cannabinoid agonists such as WIN55-212-2 and CP55-940 produce a characteristic combination of four prototypic profiles (response to the tetrad tests) including catalepsy, analgesia, hypoactivity and hypothermia.[40] These effects are reversed by the selective CB1R antagonist SR141716A (rimonabant), providing evidence for the involvement of CB1R-related mechanisms.[41] Although, many cannabinoid receptor ligands show only little or modest selectivity for both CBRs, a number of synthetic compounds are known to have significant selectivity for the CB2 receptors.[42] CB2R-selective agonists lack psychoactivity effect and so CB2 receptors are considered to

be interesting targets for treating neurological disorders.[43]

Some effects of cannabinoid receptor agonists show a biphasic behaviour that is dependent upon dose. For example, low doses of anandamide stimulated leukocyte phagocytosis and aggressive behavioural activities while high doses caused inhibitory effects on this immune function and decreased aggressiveness in mice.[44]



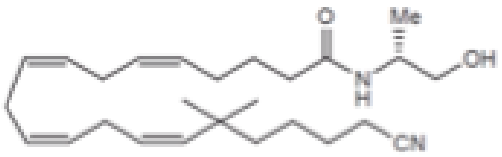
CP 55,940



WIN 55,212-2

Agonist of CB1R

R-(+)-metanandamide

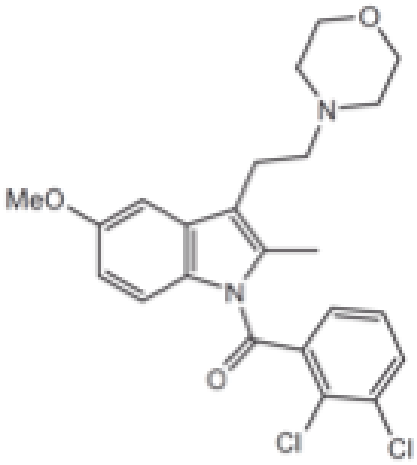


O-1812

ACEA

ACPA

Agonist of CB2R



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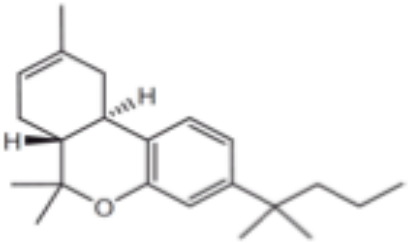
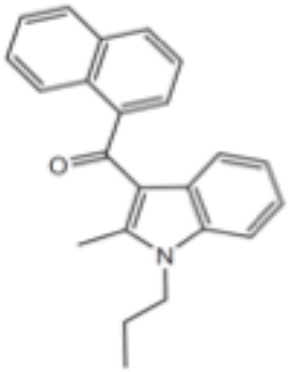
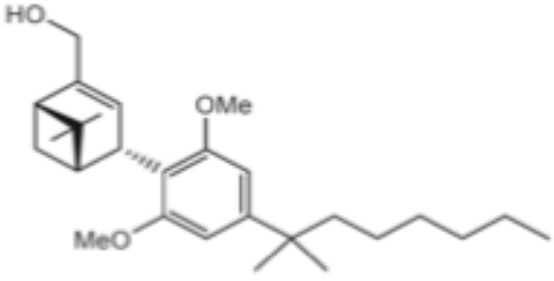


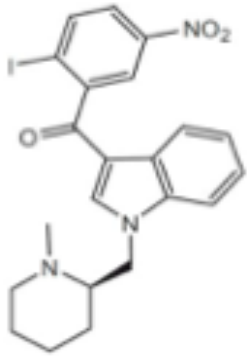
JWH-133



JWH-015



HU-308



AM-1241

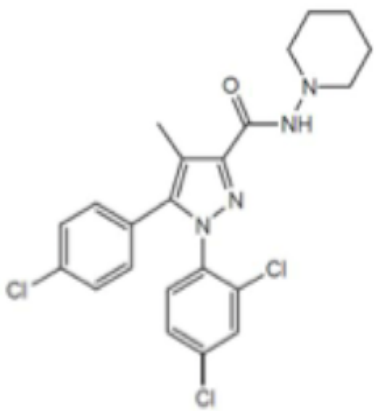
Antagonists

The first specific cannabinoid antagonist was SR141716A.[42] It blocks the actions of various cannabinoid agonists in vivo. This compound is a pure antagonist at low (nanomolar) concentrations, with higher potency and selectivity for CB1 than CB2 receptors. Even if SR141716A is CB1R-selective, it is not CB1R-specific and it blocks both CB1 and CB2 receptors at sufficiently high doses.[45]

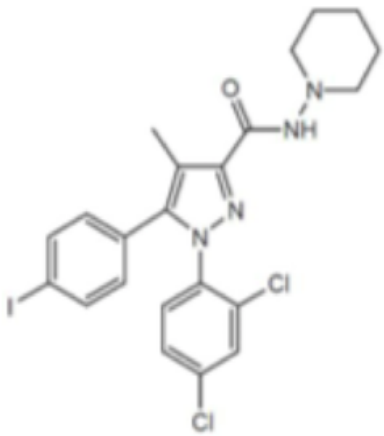
When administered the above mentioned antagonists, with cannabinoid receptor they may behave as inverse agonists in several bioassay systems. This means that they not only block the effects of endocannabinoids but also produce effects that are opposite from those produced by cannabinoid receptor agonists and suggesting that the cannabinoid system is tonically active. This tonic activity may be due to a constant release of endocannabinoids or results from a portion of cannabinoid receptors existing in a constitutively active state.[46]

Two analogues of SR141716A that have also been used to block CB1 receptor-mediated effects are AM251 and AM281. On the other hand, AM630 is a CB2R-selective antagonist/inverse agonist. It has been shown to potently reverse CP55940-induced inhibition.[47]

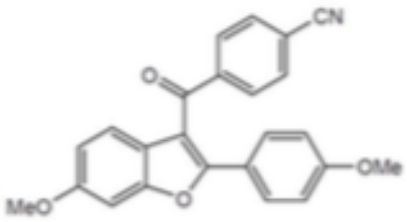
Antagonist of CB1R



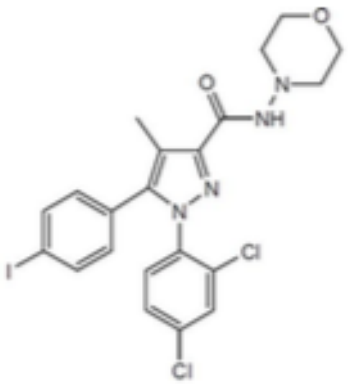
SR141716A



AM251

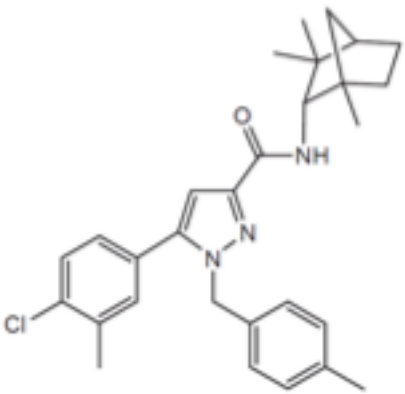


LY320135

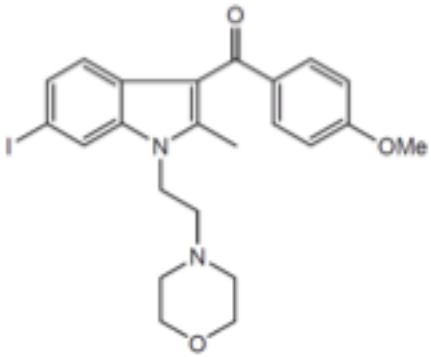


AM281

Antagonist of CB2R



SR144528



AM630

Introduction to Experimental Part

Brief Introduction to the Endocannabinoid System

Cannabis sativa L. (cannabis) is an annual dioecious plant belonging to the family Cannabaceae. Cannabis has a long history of human use as a medicinal plant, intoxicant, and ritual drug.[1] Chemical analysis of this plant in the 1940s and 1960s have revealed the presence of a group of terpeno-phenolic metabolites, known as cannabinoids, of which *trans*-Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) are the best characterized.[2]

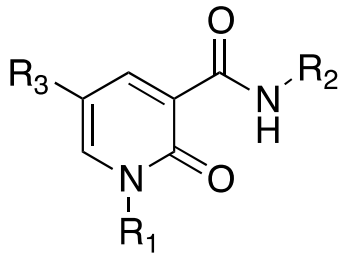
In the early 1990s two G-protein coupled receptors were identified as clearly responsible for mediating Δ^9-THC activity. Now these two receptors are known as cannabinoid receptors, type 1 (CB1R) and type 2 (CB2R). They have been cloned and characterized from mammalian tissues. CB1R is abundantly expressed in the central nervous system (CNS), but it is also localized outside the brain for example in liver, muscle, the gastrointestinal tract, heart, adrenal gland, testis, uterus and prostate. CB2R is mainly present in cells of immune system, but it has been found in low concentration in brain.[48] Cannabinoid receptors and endocannabinoid ligands (anandamide and 2-arachidonoylglycerol), together with enzymes implicated in endocannabinoid biosynthesis, degradation (MAGL and FAAH) and transport constitute the endocannabinoid system (ECS).[4] In the last years, this system has undergone to an expansion ("endocannabinoidome") since secondary receptors, ligands, and ligand metabolic enzymes were individuated. Recently researchers have observed that the effects of the two principal endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), can be enhanced by ‘‘entourage compounds’’ that inhibit their hydrolysis

via substrate competition, and thereby prolong their action. “Entourage compounds” include N-palmitylethanolamide (PEA), N-oleoylethanolamide (SEA), and *cis*-9-octadecenoamide (OEA, oleamide).[11] Moreover, recently, EMT (Endocannabinoid Membrane Transporter) was discovered as the putative cell membrane transporter that controls the bidirectional movement of 2-AG and AEA across plasma membrane. Since indirect agonistic modulation of the endocannabinoid system provides a promising therapeutic strategy during several pathological conditions, potentiation of anandamide and 2-AG signaling trough inhibition of their cellular uptake could represent an innovative possibility.[10]

The endocannabinoid system is involved in several physiological and pathological processes including cancer, appetite, fertility, memory, neuropathic and inflammatory pain, obesity, neuroprotection and neurodegenerative diseases for example Alzheimer’s disease, multiple sclerosis etc. For these reasons, the development of CBR ligands has attracted the interest of different research groups, which paid particular attention to the design of selective CB2R ligands, avoid of CB1R-mediated psychoactive effects.[49]

Structural Design

Previously in a research program aimed at obtaining CB2R selective ligands, a series of 2-oxo-1,2-dihydropyridine-3-carboxamide derivates (compounds with general structure **A**, **Figure 15**) was developed in the laboratory, where I performed my thesis work. [50,51]



A

R_1 = ethylmorpholine, *p*-fluorobenzyl;
R_2 = 4-methylciclohexyl, cycloheptyl;
R_3 = phenyl, *p*-methoxyphenyl, *p*-fluorophenyl.

Figure 15. *2-oxo-1,2-dihydropyridine-3-carboxamide derivates A.*

These compounds presented a *p*-fluorobenzyl or an ethylmorpholine moiety in position 1 and an alkyl amide in position 3 of the pyridine ring and they were non-substituted or substituted at position 5.

Derivatives with general structure **A** showed potent and selective affinity towards CB2 receptors. Interestingly it was discovered that the substituent in position 5 of the pyridine ring was the main responsible for the functional activity of this class of compounds.[51] In particular, replacing hydrogen or bromine atom in position C5 with a phenyl group and with a *p*-methoxyphenyl group, CB2R activity shifts from agonism to inverse agonism and neutral antagonism, respectively (**Figure 16**).

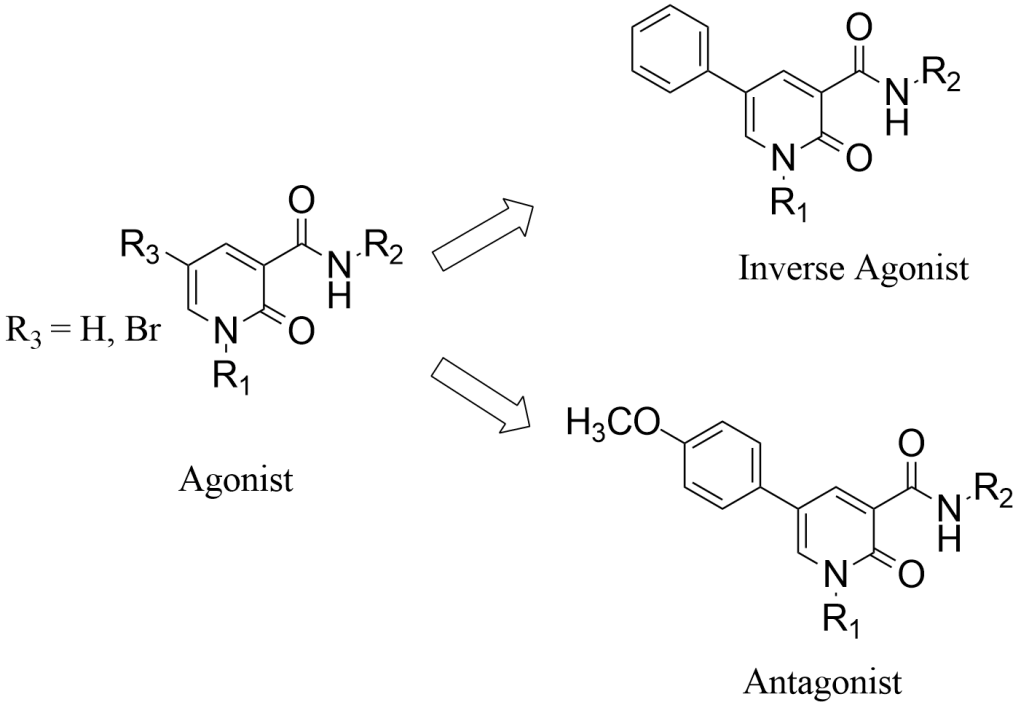


Figure 16. *Substituent in position 5 is the main responsible for the functional activity of the class of 2-oxo-1,2-dihydropyridine-3-carboxamide derivatives.*

After, in order to investigate the structure-activity relationship within this class of compounds, a series of 6-methyl-2-oxo-1,2-dihydropyridine-3-carboxamide derivatives (compounds with general structure **B**, **Figure 17**) was developed by inserting a methyl group in position 6 of the 2-oxo-1,2-dihydropyridine ring to evaluate the influence of a small substituent in this position on CBR affinity.

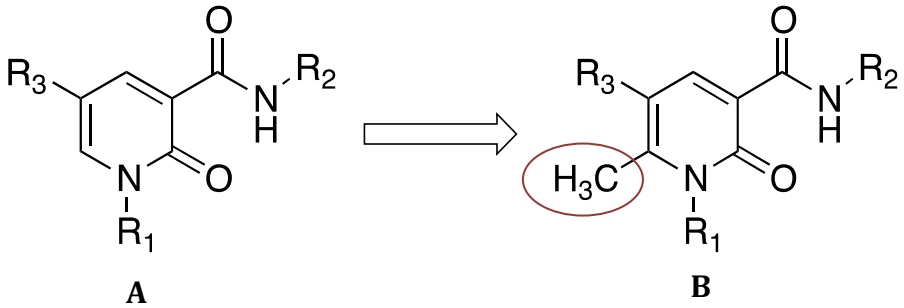
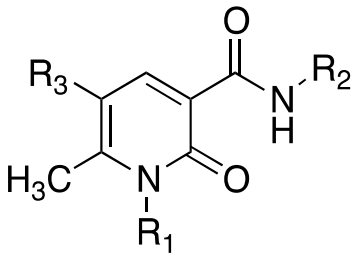


Figure 17. *Design of compounds with general structure B.*

Substituents in position 1 (R_1 in **Figure 18**) and in position 5 (R_3 in **Figure 18**) and aliphatic carboxamide in position 3 (R_2 in **Figure 18**) of compounds with general structure **B**, have been chosen on the basis of the best results obtained for previous derivatives with general structure **A**.



B

R_1 = *p*-fluorobenzyl, ethylmorpholine;
R_2 = cycloheptyl;
R_3 = H, Br, phenyl, *p*-methoxyphenyl.

Figure 18. *6-methyl-2-oxo-1,2-dihydropyridine-3-carboxamide derivatives B.*

The insertion of a methyl group in position C6 does not significantly affet neither the binding of 2-oxopyridine-1,2-dihydropyridine-3-carboxamide derivatives **A** nor the influence of the substituent in C5 position on the functional modulation of CB2R.

Coherently with these results, to further deepen the structure-activity relationship of this class of compounds, during my thesis work I modified the 2-oxo-1,2-dihydropyridine-3-carboxamide nucleus following three different approaches:

1) Shift of the methyl group of compounds **B** from 6 to 4 position of the pyridine ring to obtain 4-methyl-2-oxo-1,2-dihydropyridin-3-carboxamide derivatives **C** and **D** (**Figure 19a)**.

2) Shift of the aromatic substituents of compounds **A** from 5 to 4 position of the pyridine ring to obtain the correspondent 4-aryl-2-oxo-1,2-

dihydropyridin-3-carboxamide derivatives **E** (**Figure 19b**).

3) Insertion of a further substituent in position 4 of the pyridine ring of compounds **A** to obtain **F** derivatives (**Figure 19c**).

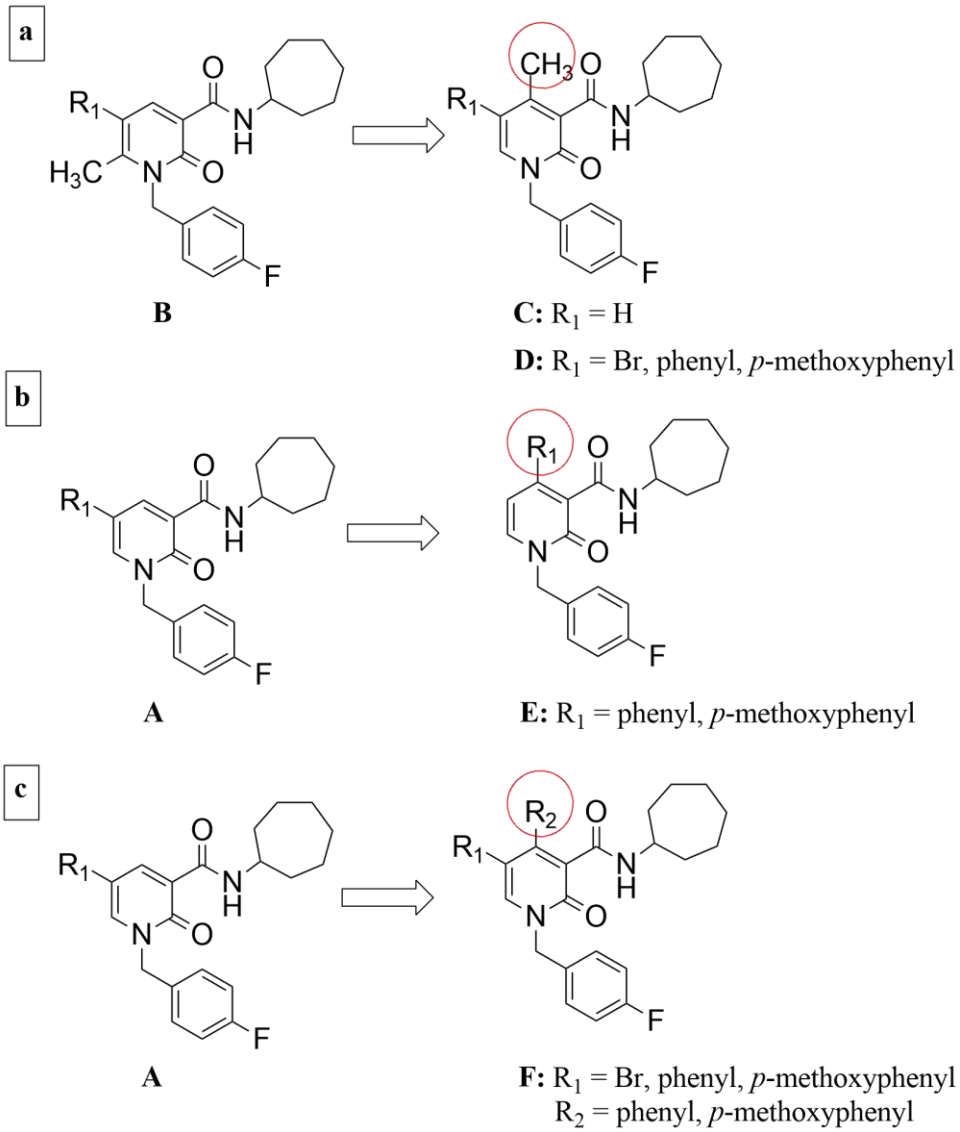
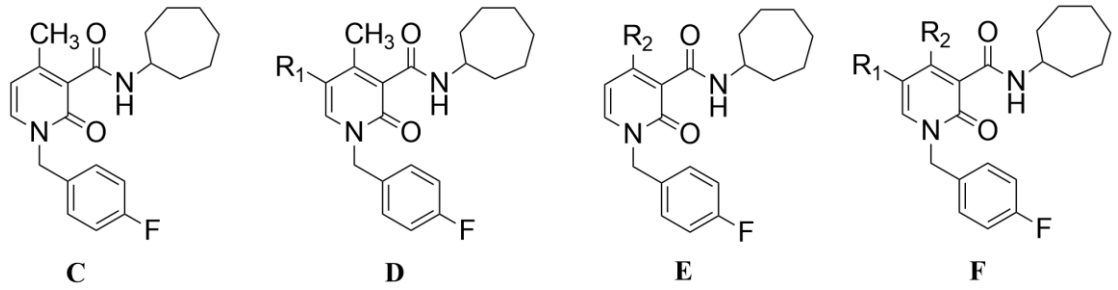


Figure 19. ***a)*** *4-methyl-2-oxo-1,2-dihydropyridin-3-carboxamide derivatives* ***C*** *and* ***D****;* ***b)*** *6-aryl-2-oxo-1,2-dihydropyridin-3-carboxamide derivatives* ***E****;* ***c)*** *derivatives with general structure* ***F****.*

Thus, I designed and synthesized compounds with general structure **C**, **D**, **E** and **F** (**Figure 20**).



R_1 = Br, phenyl, *p*-methoxyphenyl
R_2 = phenyl, *p*-methoxyphenyl

Figure 20. *Compounds with general sturctures **C**, **D**, **E**, and **F**.*

All the substituents have been chosen on the basis of the best results obtained with the previous compounds. Therefore, all the synthesized compounds are characterized by the presence of a *p*-fluorobenzyl group in position 1 of the pyridine nucleus and a cycloheptyl moiety at the 3-carboxamide nitrogen. Moreover, hydrogen, bromine, phenyl and *p*-methoxyphenyl are the substituents chosen for the position 5 (R_1), of compounds **D** and **F** whereas the phenyl and the the *p*-methoxyphenyl moieties characterized the position 4 of compounds **E** and **F**.

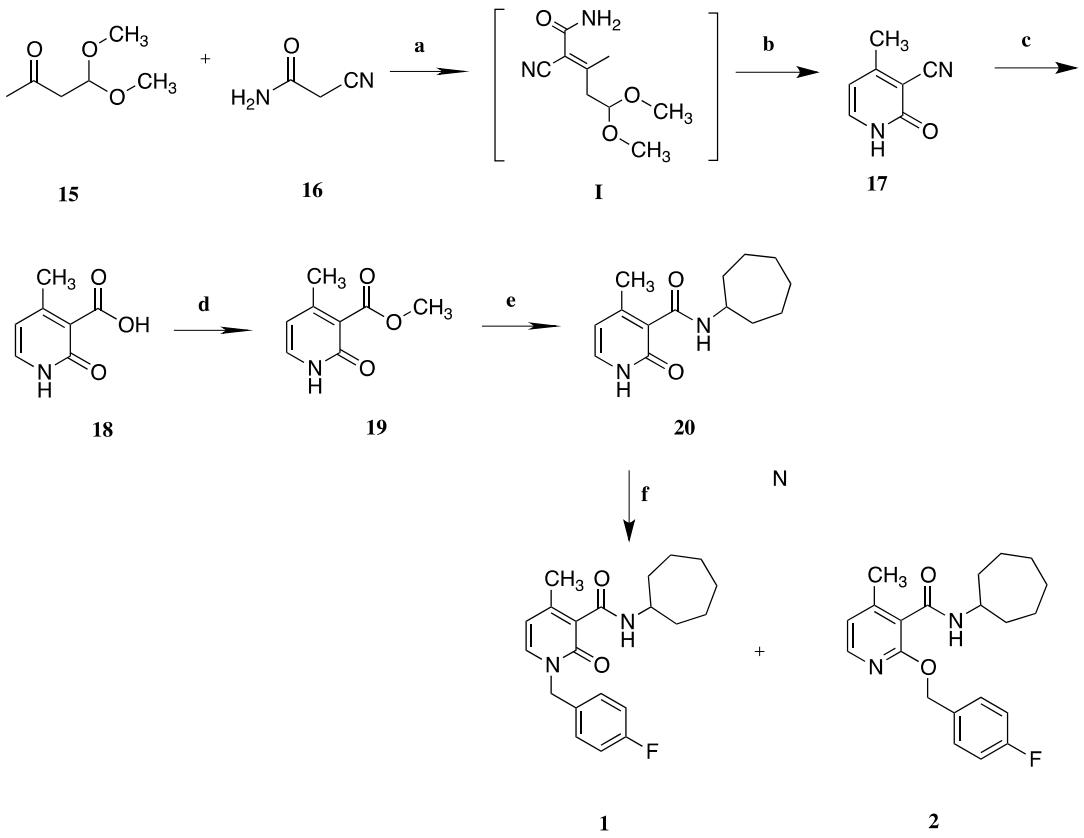
Synthetic Chemistry

Synthesis of 4-methyl-2-oxo-1,2-dihydropyridine derivatives

The series of 4-methyl-2-oxo-1,2-dihydropyridine derivatives **C** and **D** (Figure 19a) derived from the shifting of the methyl group of compounds **B** from 6 to 4 position of the pyridine ring.

Compounds with general structure **C** were prepared following the synthetic route shown in **Scheme 1**.

Scheme 1. Synthetic procedure for compounds C.

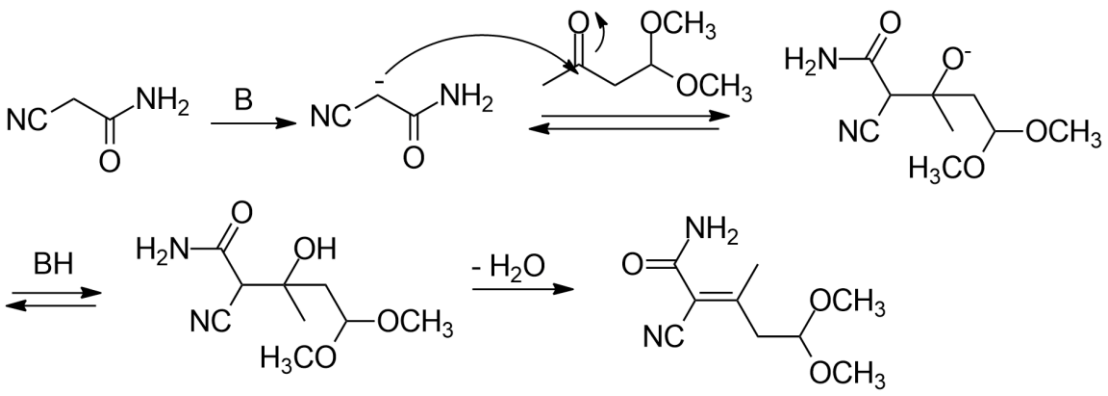


Reagents and conditions: (a) NH_4OAc, AcOH, toluene, reflux, 116 °C, 8 h; **(b)** conc. H_2SO_4, 50 °C, 3 h; **(c)** 50% H_2SO_4, reflux, 120 °C, 8 h; **(d)** MeOH, conc. H_2SO_4., MW, 110 °C, 50 min, (200 W, 100 psi) or reflux, 85 °C, overnight; **(e)** cycloheptylamine, MW, 130 °C, 30 min, (140 W, 100 psi) or reflux, 150 °C, 48 h; **(f) 1)** CsF, dry DMF, 1h, r.t.; **2)** 4-fluorobenzyl chloride, r.t, overnight.

The key compound of this synthetic route is 3-cyano-2-hydroxy-4-methylpyridine **17**. This derivative was obtained *via* a two-step procedure.[3] Firstly, a Knoevenagel condensation of 4,4-dimethoxyl-2-butanone **15** with 2-cyanoacetamide **16**, in presence of the mixture 4 wt% ammonium acetate/30 wt% acetic acid as catalyst and using anhydrous toluene as solvent, afforded intermediate **I**.

The possible mechanism of the condensation of **15** with **16** is reported in **Scheme 2**.[52]

Scheme 2. Possible mechanism of the Knovenagel condensation of 15 with 16.

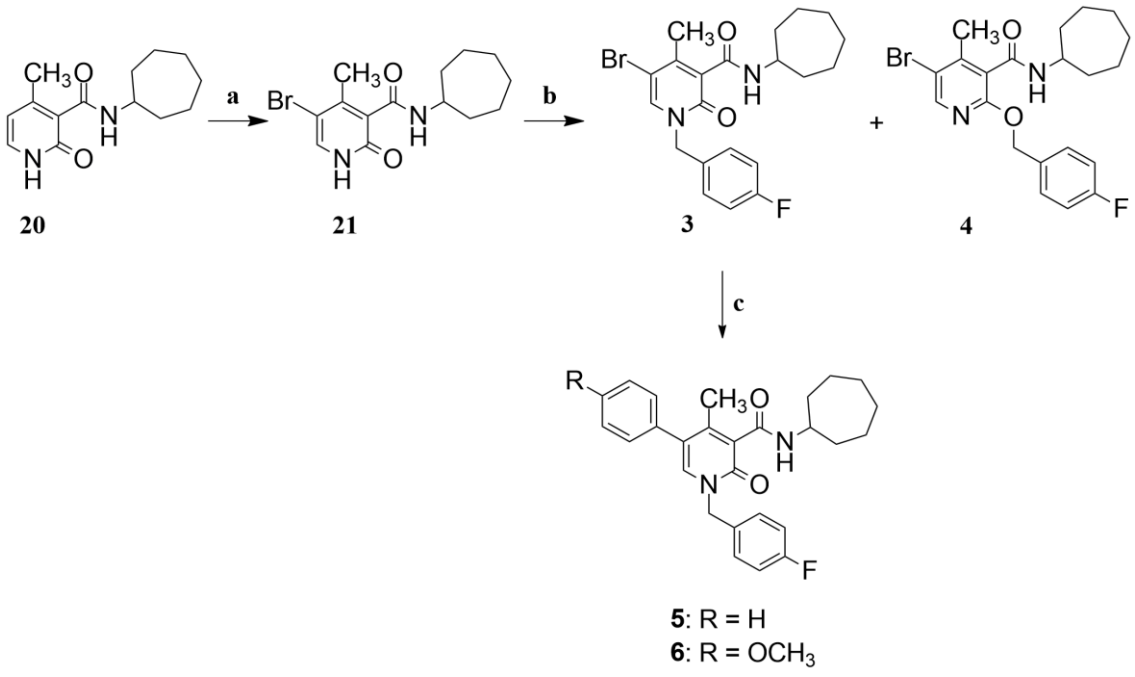


Since the mechanism of the condensation involves lose of water, an azeotropic distillation was adopted to remove it from the reaction environment. After, intermediate **I**, not isolated because of its high boiling point,[52] was directly hydrolyzed to aldeide under acid conditions, and then condensed with the amino to afford 3-cyano-2-hydroxy-4-methyl-pyridine **17**. This reaction was performed using concentrated H_2SO_4 and heating the mixture at 50° C for 3 h.[52] Then compound **17** was then hydrolyzed to the corresponding carboxylic acid derivative **18** by heating at 120 °C with 50% H_2SO_4.[53] The carboxylic acid **18** was treated with concentrated H_2SO_4 and MeOH and heated at 100° C in a microwave reactor (CEM) for 50 minutes (200 W, 100 psi) [50] or refluxed at 85° C, overnight, to give the corresponding methyl ester **19**, which was reacted with cycloheptylamine at 130 °C in a microwave reactor (CEM) for 30 minutes (140

W, 100 psi) or under reflux at 150° C for 48 h to afford the carboxamide derivative **20**.[50] Finally, compound **20** was treated with CsF in anhydrous DMF, at 25 °C, and, after 1 h, added of *p*-fluorobenzyl chloride,[54] giving the desired N-alkylated derivative **1.** 2-(*p*-fluorobenzyloxy)pyridine derivative **2** was also obtained, therefore separation by chromatography of the two positional isomers was necessary. The structure of compounds **1-2** was verified by ^{1}H-NMR analysis. The two isomers was easly distinguished since the benzylic methylene protons attached to the oxygen atom are more deshielding, therefore they display a resonance signal (singulet) that is higher field than the two benzylic methylene protons bonded to the nitrogen atom.

Preparation of compounds with general structure **D** started from compound **20** (**Scheme 3**) which was treated with a solution of bromine in $CHCl_3$ [55] affording the corresponding brominated derivative **21**.

Scheme 3. Synthetic procedure for compounds with general structure D.



Reagents and conditions: (a) Br_2, $CHCl_3$, r.t, overnight; **(b) 1)** dry DMF, CsF, r.t, 1 h; **2)** *p*-fluorobenzyl chloride, r.t, overnight; **(c) 1)** dry toluene, PPh_3, $Pd(OAc)_2$, r. t, 15 min, **2)** anh. K_2CO_3, proper arylboronic acid, 100 °C, overnight.

After, the desired N-alkylated derivative **3** was obtained by treatment of **21** with *p*-fluorobenzyl *via* a procedure similar to those used for previous compounds (**1-2**).[54] Also in this case, a chromatography separation of the two positional isomers **3** and **4** was necessary.

5-Aryl-2-oxo-1,2-dihydropyridine-3-carboxamide compounds **5** and **6** were obtained starting from the 5-bromo derivative **3** *via* a Suzuki-Miyaura cross-coupling reaction[56] (**Figure 21**) with phenylboronic acid or *p*-methoxyphenylboronic acid in dry toluene using tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) as catalyst and anhydrous potassium carbonate as base, refluxing at 100 °C overnight.[57] Palladium(0) compound required in this cycle, $Pd(PPh_3)_4$, was prepared *in situ* from a palladium(II) precursor using triphenylphosphine (PPh_3) and palladium acetate ($Pd(OAc)_2$).
Compounds **5** and **6** were then purified by flash column chromatography.

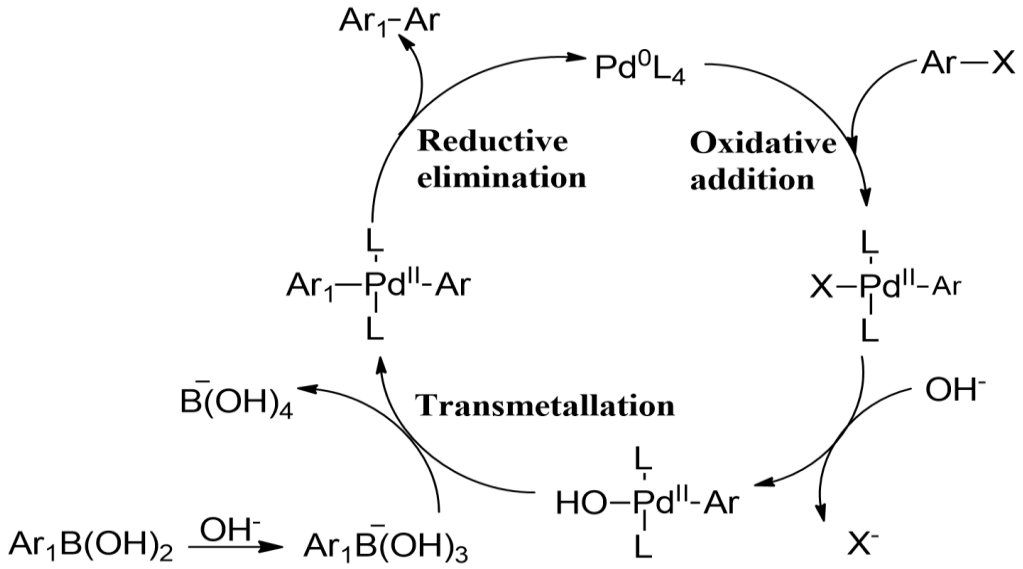


Figure 21. *Suzuky cross-coupling reaction mechanism.*[56]

Synthesis of 4-aryl-2-oxo-1,2-dihydropyridine derivatives

Compounds with general structures **E** (**Figure 19b**) and **F** (**Figure 19c**) share the presence of an aromatic substituent (phenyl or *p*-methoxyphenyl) in position for of the pyridine nucleus.

During my thesis work, several efforts have been made to prepare compounds with general structure **E**, **7-8**. Initially the synthesis of these compounds was planned starting from 4-aryl-1-(4-fluorobenzyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile which, after being hydrolyzed to the corresponding carboxylic acid, would be easily converted into the desired carboxamide derivatives **7** and **8** (**Figure 22**).

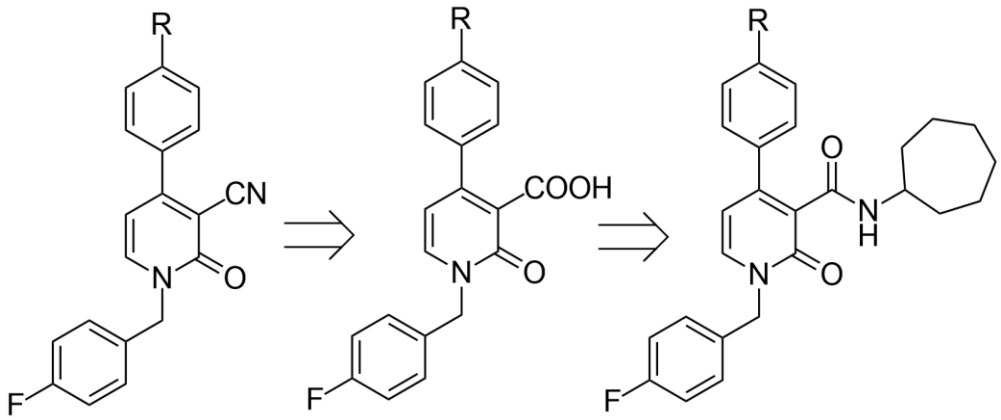
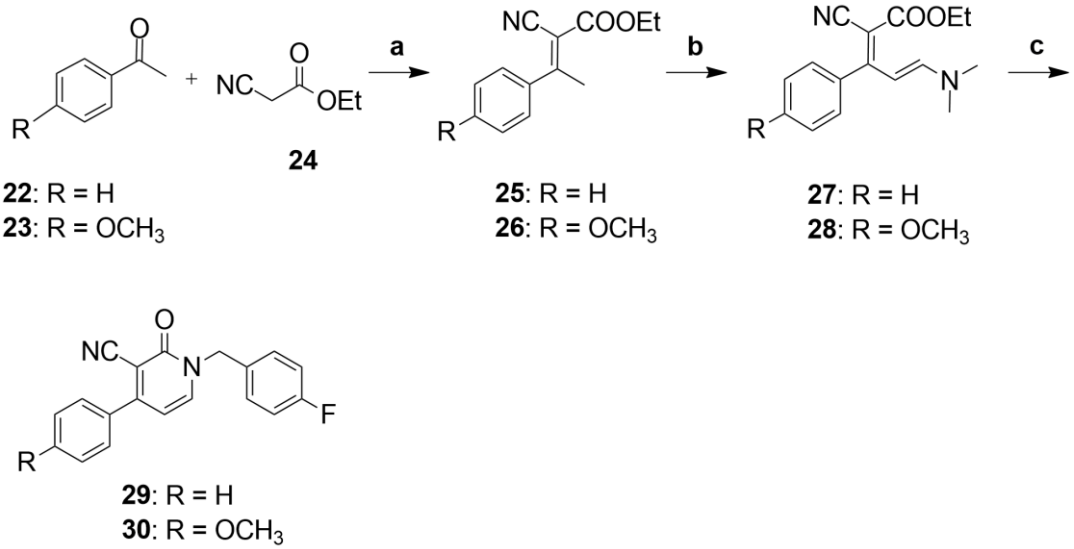


Figure 22. *Initial synthetic plan for preparation of compounds with general structure **E**.*

3-cyano-2-pyridones **29-30** were easly obtained using modified Kibou's synthetic procedure[58] depicted in **Scheme 4**. Ethyl 2-cyano-3-arylbut-2-enoate (**30-31**) were prepared by a Lewis acid-mediated Knoevenagel condensation[59] of the opportune aromatic ketone (acetophenone **22** or *p*-methoxyacetophenone **23**)

with ethyl cyanoacetate **24** in presence of titanium(IV) chloride and pyridine in dichloromethane. After, the reaction between α,β-unsaturated compounds **25**-**26** and *N,N*-dimethylformamide-dimethylacetal (DMF-DMA) under solvent-free conditions, at room temperature afforded the corresponding enaminonitriles **27**-**28**. Finally, cyclyzation of the enaminonitriles **27**-**28** with *p*-flurobenzylamine, at 90 °C led to 3-cyano-2-pyridones **29**-**30**.[58]

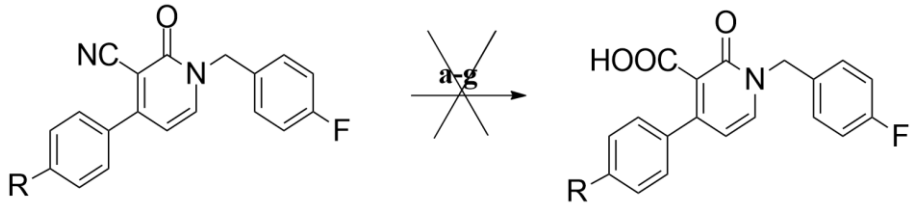
Scheme 4. Synthetic procedure for compound 3-cyano-2-pyridones.



Reagents and conditions: (a) $TiCl_4$, pyridine, CH_2Cl_2, 0 °C to r.t., overnight; **(b)** DMF-DMA, solvent-free, 24 h; **(c)** *p*-fluorobenzylamine, solvent-free, 90 °C, 2 h.

Then, several attempts have been made to hydrolyze 3-cyano-2-pyridones **29-30** to the corresponding carboxylic acid derivatives (**Scheme 5**). Firstly, a procedure[53] similar to that used for converting 4-methyl-2-oxo-1,2-dihydropyridine-3-carbonitrile **17** into its carboxylic acid derivative **18** (**Scheme 1**) was carried out, treating compounds **29-30** with 50% H_2SO_4 at 120 °C for 8h,

Scheme 4. Synthetic procedure for compound 3-cyano-2-pyridones.



29: R = H
30: R = OCH_3

Reagents and conditions: (a) 50% H_2SO_4, 120 °C, 8h; **(b)** (60%, 70% or 98%) H_2SO_4, 130-160 °C, 8-24 h; **(c)** 6N HCl, reflux, 3 h; **(d)** concentrated HCl, reflux, 48 h; **(e)** 60% KOH/EtOH, reflux, 16 h; **(f)** 20% aq. NaOH, reflux, 26 h or MW, 140 °C; **(g) 1)** 30% aq. H_2O_2, 10% NaOH, EtOH, 23 °C to 50 °C, 18 h; **2)** 10% KOH, reflux, 20 h.

but it failed and the starting material was recovered. Then, the same reaction was performed using H_2SO_4 of different concentrations (60%, 70% or 98%) and/or increasing the temperature and the reaction time, but unsuccessfully. Further reactions under acidic conditions were performed heating the nitrile derivatives with 6N HCl for 3 h[60] or with concentrated HCl for 48 h, [61] but these tries were unfruitful once again. Since all the attempts of hydrolysis under acidic conditions failed, all the subsequent tries were made using basic conditions. Thus, nitrile derivatives **29-30** were treated with 60% ethanolic KOH refluxing for 16 h,[62] and also with 20% aqueous NaOH refluxing for 24 h[63] or heating at 140 °C in a microwave reactor,[64] but in both the cases hydrolysis of the cyano group stopped at the intermediate amide stage (**Figure 23**).

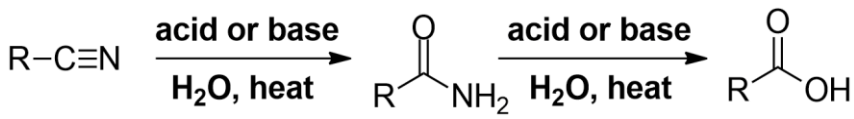


Figure 23. *Hydrolysis of nitrile derivatives under acid or basic condition occur via amide.*

The last attempt was performed by a two-step procedure, which involved initial peroxide-mediated conversion of the nitrile mojety to the corresponding primary amide[65, 66] and the subsequent extended exposure of the resulting amide to refluxing aqueous KOH, [68] but also this procedure gave unsatisfactory results.

The reasons of this hydrolysis resistance that characterized the nitrile derivative **29**-**30** rather than the nitrile compound **17**, is probably correlated to the nature of the substituents in "beta" to the nitrile moiety (C4 of the pyridine ring)(**Figure 24**).

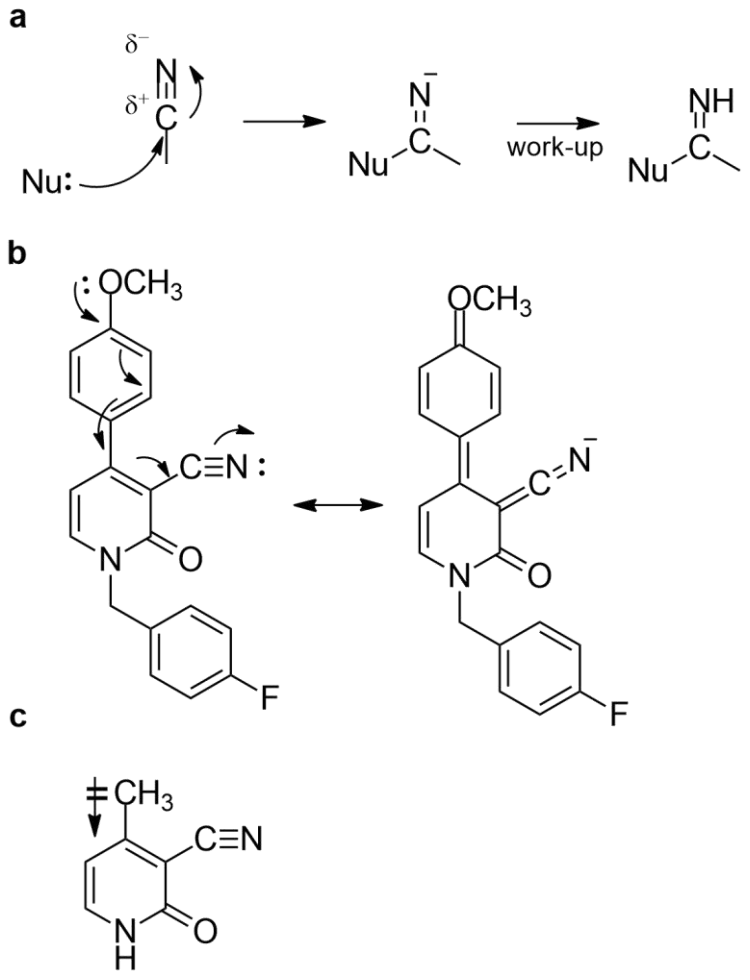
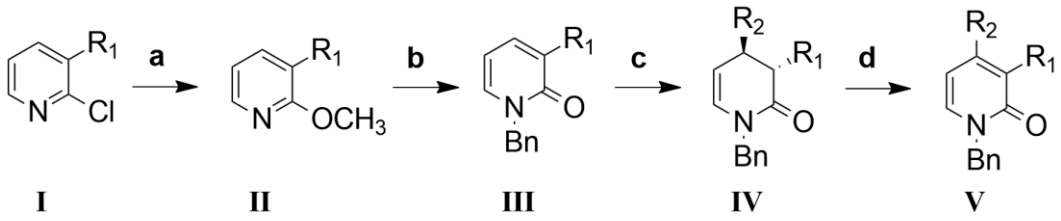


Figure 24. ***a)*** *Carbon atom in nitrile moiety has a partial positive charge;* ***b)*** *Aromatic substituents exert an electro-donating effect toward nitrile carbon atom, whereas* ***c)*** *methyl group exert electro-donating inductive effect.*

Indeed, the phenyl group and, even more, the *p*-methoxyphenyl group exert electro-donating effects, since they are able to delocalize the partial positive charge of carbon atom in nitrile (**Figure 24a-b**), stabilizing it and, consequently, making the nitrile partially positive carbon less reactive toward the nucleophilic attack of water molecules. The methyl group in position C4 of derivative **17** exert only an electron-donating inductive effect (**Figure 24c**) that is certainly far less strong than electron-donating mesomeric effect.

For all these reasons all the attention were focused on a recent procedure for obtaining the 2-pyridone scaffold with an aromatic substituent in position 4, developed by Krawczyk et al.[67] This synthetic route is depicted on **Scheme 5** and starts from various 3-substituted-2-chloro-pyridines (general structure **I**) that treated with freshly prepared sodium methoxide under reflux conditions in methanolic solutions afford the corresponding 2-methoxy substituted pyridines (**II**) *via* a nucleophilic substitution reaction. The *N*-benzylation of the pyridine nucleus in compounds **II** and to **III** succeeds with benzyl bromide as alkylating agent in toluene under reflux conditions. The 2-methoxy function in the primary yielded *N*-benzyl pyridinium cation undergo a bond cleavage by the attack of the bromide anion so that the alkylation reaction product finally afford a pyridine-2-one structure (**III**) after the rearrangement of the pyridine nucleus bonds. The varying 3-substituted pyridine-2-ones are then treated with various grignards reagents at low temperatures, in dried THF as solvent, using copper(I) iodide as catalyst and lithium chloride to improve its the solubility, to afford the corresponding 4-aryl tetrahydropyridine-2-ones **IV** which are then rearomatized to final target structures **V** by heating in refluxing toluene with excess amounts of heat-activated manganese dioxide.

Scheme 5. Krawczyk's synthetic procedure for 4-aryl-2-pyridones derivatives.[67]



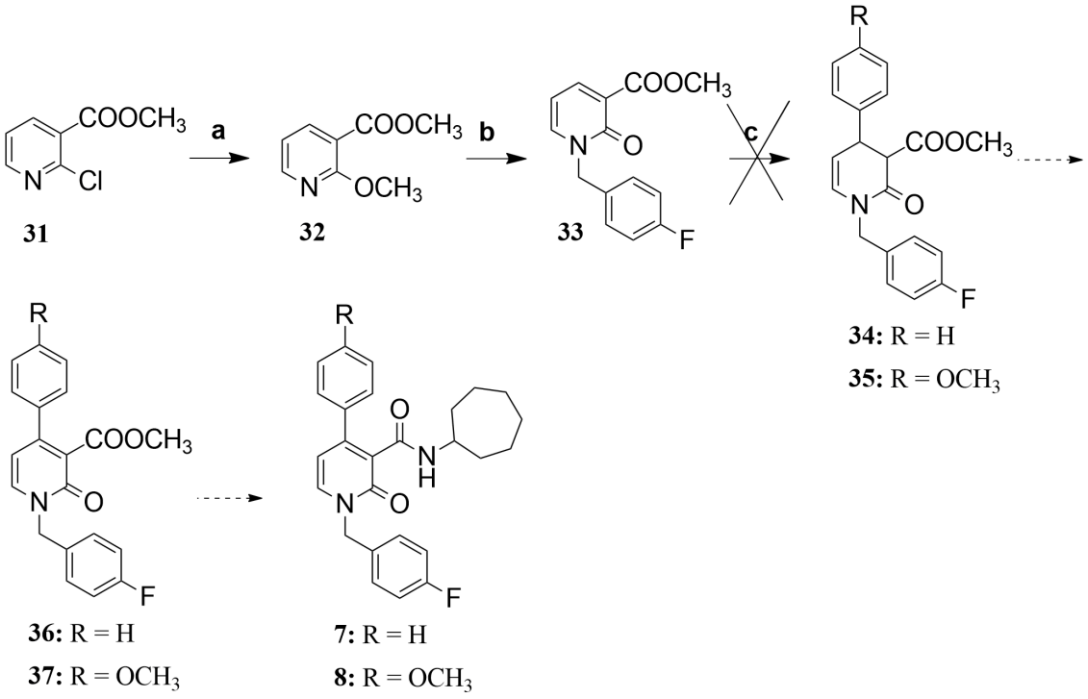
I **II** **III** **IV** **V**

R_1 = $COOCH_3$, CN, COOBn;
R_2 = Ph, Bn, 2- or 4-OMePh, 2- or 4- tolyl.

Reagents and conditions: (a) MeOH, NaOMe, reflux, 6 h; **(b)** BnBr, 120 °C, 20 h; **(c)** THF, Cu(I)I, LiCl, ArylMgBr, -40 °C to r.t, 6 h; **(d)** toluene, MnO_2, 130 °C, 6–8 h.

Initially I literally followed this synthetic route, starting from methyl 2-chloropyridine-3-carboxylate **31** (**Scheme 6**) and using *p*-fluorobenzyl bromide as alkytating in *N*-alkylation rather than benzyl bromide (compound **33**, **Scheme 6**). Therefore, once obtained the methyl 4-aryl-2-oxo-1,2-dihydropyridine-3-carboxylate scaffold, it would be converted into the desired *N*-cycloheptyl carboxamide derivatives **7-8** (**Scheme 6**). Unfortunately, the reaction with the Grignard reagent failed since a mixture difficult to purify was obtained.

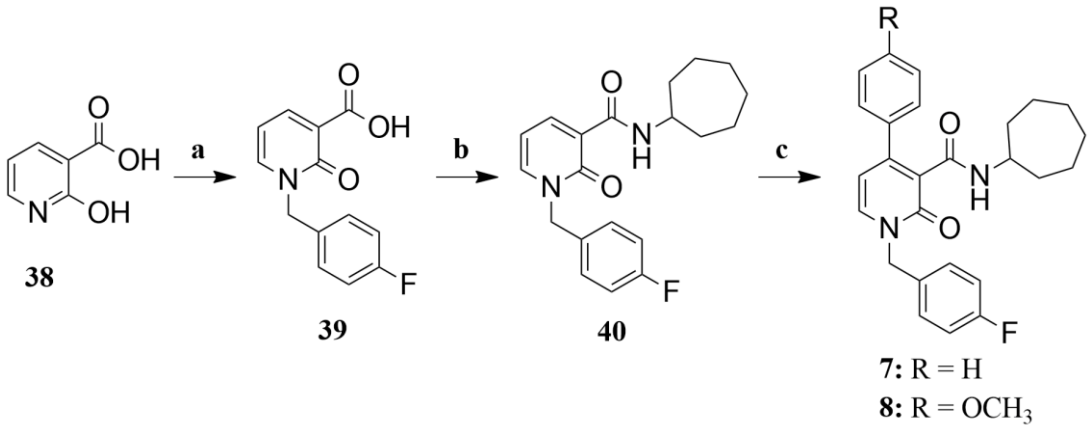
Scheme 6. Second attempt for preparation of 4-aryl-2-pyridones derivatives 7-8.



Reagents and conditions: (a) MeOH, NaOMe, reflux, 65 °C, 6 h; **(b)** *p*-fluorobenzyl bromide, 120 °C, 20 h; **(c)** dry THF, Cu(I)I, LiBr, phenylmagnesiumbromide or *p*-methoxyphenylmagnesiumbromide; -40 °C to rt, 6 h.

After, because the reported yields of the last two steps (conjugate addition and rearomatization) were less than 10%,[67] the subsequent attempts of the addition reaction with the Grignard reagents were performed directly on the *N*-cycloheptyl carboxamide derivatives rather than on the methyl carboxylate derivatives, in order to avoid a further step after the rearomatization. Thus, the *N*-cycloheptyl carboxamide derivative **40** was obtained following the synthetic route depicted in **Scheme 7**.

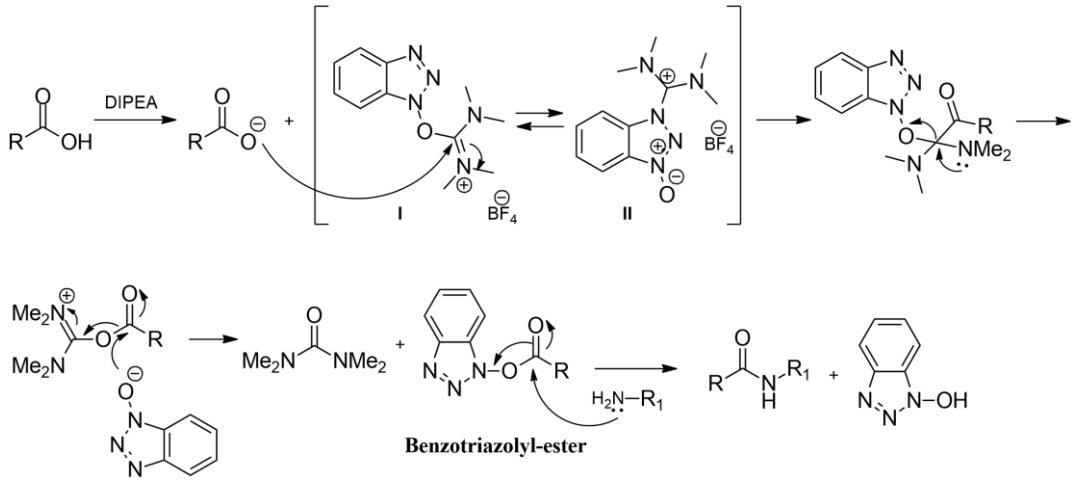
Scheme 7. Synthetic route for compounds 7-8.



Reagents and conditions: **(a) 1)** NaH, *p*-fluorobenzyl bromide, dry DMF, 50 °C, overnight; **2)** 10% NaOH, reflux, 100 °C, 4 h; **(b) 1)** dry DMF, DIPEA, TBTU, 0 °C, 30 min, **2)** cycloheptylamine, 0 °C to r.t, overnight; **(c)** Cu(I)I, LiBr, phenylmagnesium bromide or *p*-methoxyphenylmagnesium bromide, THF, -40 °C, 6 h.

N-alkylation of the commercial available 2-hydroxynicotinic **38** acid with *p*-fluorobenzyl bromide in presence of NaH, heating at 50 °C afforded the desired carboxylic acid **39** mixed with 3-benzyloxy ester substituted product as side product (not showed in th Scheme). Therefore, hydrolysis was necessary, and the mixture was refluxed at 100° C with 10% NaOH to ensure its complete conversion into the carboxylic acid derivative **39**. After, it was initially treated with the coupling agent 2-[(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate] (TBTU) at 0 °C, in presence of N,N-diisopropylethyl amine (DIPEA), to afford the corresponding activated benzotriazolyl-ester (**Scheme 8**), which was added of cycloheptylamine to give the desired carboxamide derivative **40**.

Scheme 8. Proposed mechanism for the amide bond formation through TBTU



Finally, compound **40** was treated with the proper Grignard reagent (phenylmagnesium bromide or *p*-methoxyphenylmagnesium bromide) under the same conditions described by Krawczyk[67]: using copper(I) iodide and lithium bromide in dried THF, at -40 °C. Surprisingly, instead of obtaining the corresponding 4-aryl tetrahydropyridine-2-one derivatives as described by Krawczyk[67], after the purification of the crude residue by flash chromatography, the rearomatizated compounds **7-8** were obtained directly. Structure of compounds **7-8** was verified by ^{1}H-NMR analysis. The spectra showed the absence of the signal of the aliphatic hydrogen atom at C3 and C4, and the consequent presence of the aromatic system (**Figure 25a and 25b**).

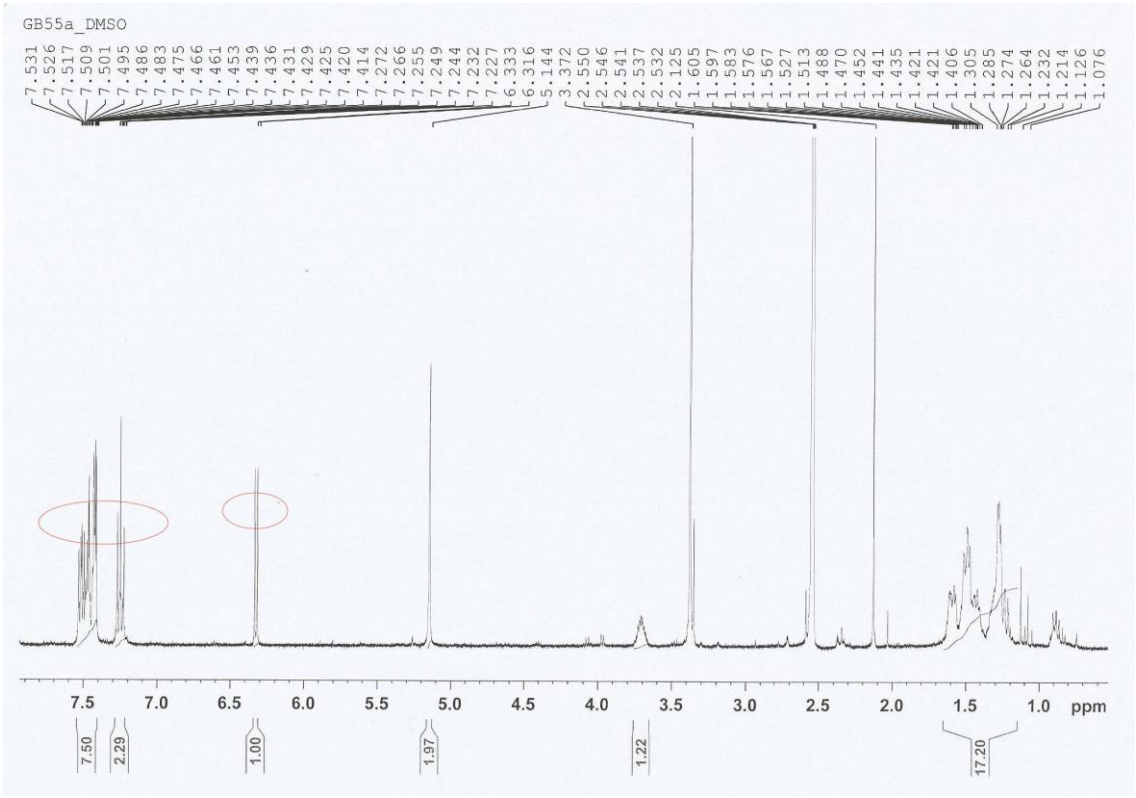


Figure 25a. *^{1}H-NMR spectrum of compound 7.*

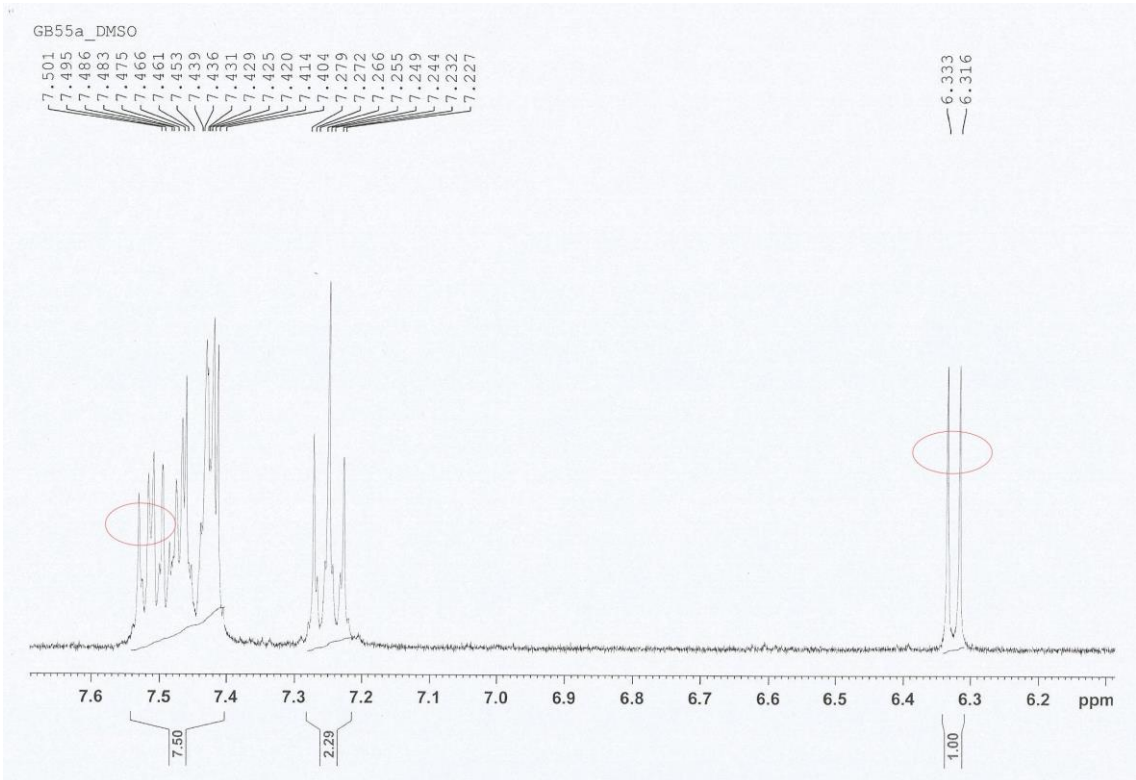
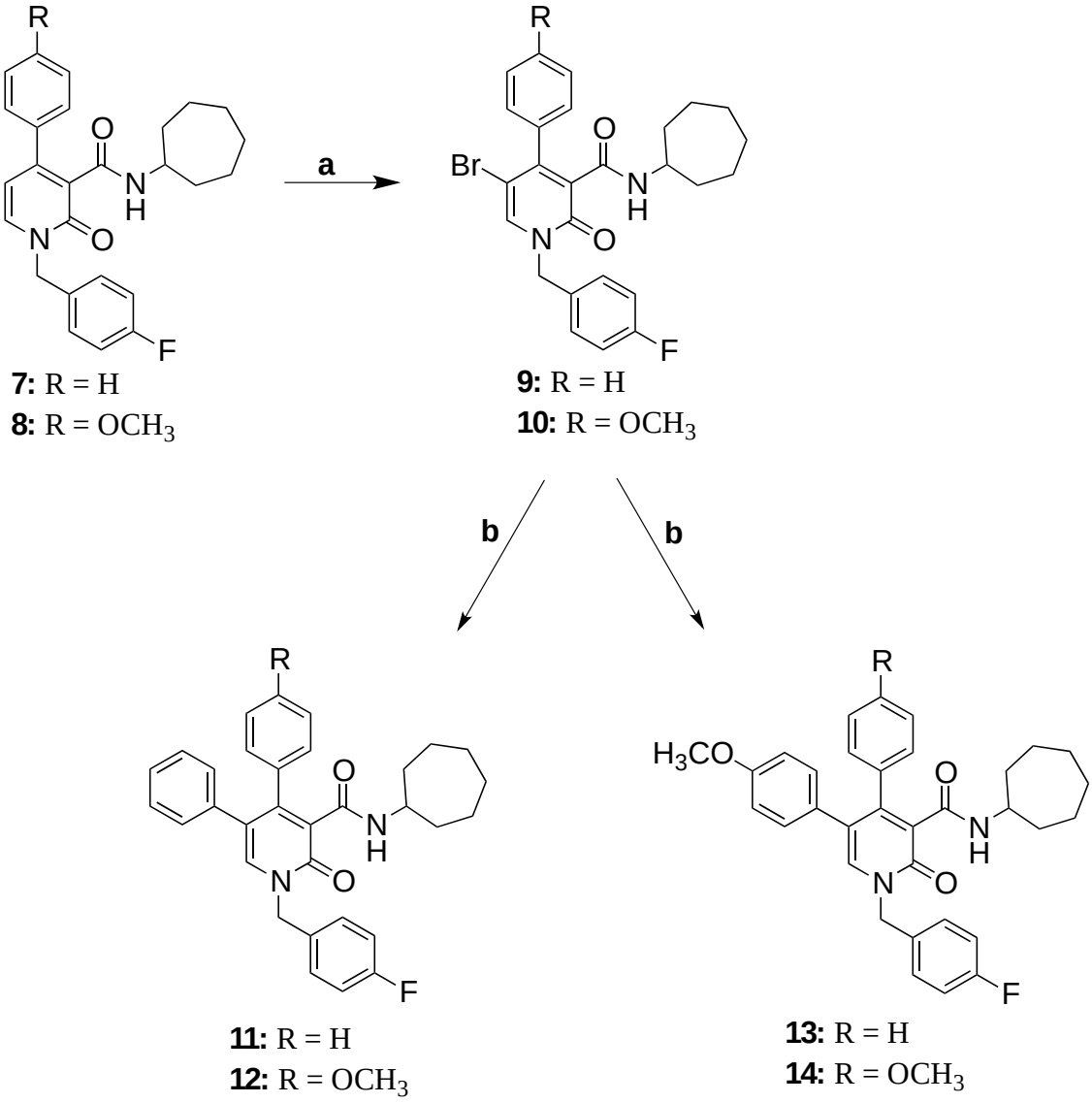


Figure 25b. *Enlargement of aromatic signals in ^{1}H-NMR spectrum of compound 7.*

Compounds with general structure **F** were prepared starting from compounds **7-8.** The synthetic pathway is showed in **Scheme 9**.

Scheme 9. Synthetic pathway for compounds with general structure F.



7: R = H
8: R = OCH_3

9: R = H
10: R = OCH_3

11: R = H
12: R = OCH_3

13: R = H
14: R = OCH_3

Reagents and conditions: **(a)** Br_2, $CHCl_3$, r.t, overnight; **(b) 1)** dry toluene, PPh_3, $Pd(OAc)_2$, r. t, 15 min; **2)** anh. K_2CO_3, proper arylboronic, 100 °C, overnight.

Compounds **7-8** were treated with a solution of bromine in $CHCl_3$ to afford the desired compounds **8-9** which were purified by trituration with high boiling point petroleum ether. After, to obtained compounds **10-13,** derivatives **8-9** were submitted to a Suzuki cross-coupling reaction under the same conditions previously described for compounds **5-6**. Thus, toluene, triphenylphosphine and palladium acetate were mixed together to afford the catalyst tetrakis(triphenylphosphine)palladium(0). After, the catalyst was heated at 100 °C overnight with potassium carbonate, compound **8** or **9**, and with phenylboronic acid to afford compounds **10** and **12**, or with p-methoxyphenylboronic acid, to afford compounds **11** and **13**.

Experimental part

Commercially available reagents were purchased from Sigma Aldrich or Alfa Aesar and used without purification. ^{1}H-NMR spectra were recorded on a Bruker AVANCE III™ 400 spectrometer (operating at 400 MHz). Chemical shift (δ) are reported in parts per million related to the residual solvent signal, while coupling constants (*J*) are expressed in Hertz (Hz). Microwave-assisted reactions were run in a CEM microwave synthesizer. Evaporation was carried out under vacuum using a rotating evaporator. The system for isocratic flash chromatography includes glass chromathography columns and silica gel 60 Å (0.040-0.063 mm; MERK) or a Buchi® Pump Module C-601 (continuous flow of solvents up to 250ml/min at max 10bar) and Buchi® prepacked cartridges (silica gel 60, particle size 40-63 μm). Reactions was monitored by TLC on Merck aluminium silica gel (60 F_{254}) plates that were visualized under a UV lamp (λ = 254 nm).

2-*Hydroxy-3-cyano-4-methylpyridine* (17)

Commercial available 4,4-dimethoxyl-2-butanone **15** (2.0 mL, 15.13 mmol) and cyanoacetamide **16** (1.6 g, 18.92 mmol), together with ammonium acetate (79.3 mg), acetic acid (0.6 mL) and dry toluene (12.4 mL) were refluxed at 116° C with stirring for 8 h. Water generated was removed through azeotropic distillation using a Dean-Stark apparatus. After toluene was distilled off under reduced pressure at 50 °C. The residue, intermediate I, was then cooled to room temperature and EtOH (6.9 mL) was added at 25 °C, followed by the slow addition of concentrated H_2SO_4 (2.6 mL). The mixture was heated at 50 °C and stirred for 3 h. The content was cooled to 5 °C with an ice-water bath. Water (1.3 mL) was added slowly and the reaction was left in the ice-water bath until no more precipitate was formed. Then, the mixture was filtered under vacuum, washed with ethanol and dried to give 903.9 mg (6.74 mmol) of brown solid **17**.

Yield: 45%

^{1}H-NMR: (DMSO, 400 MHz) δ (ppm) 2.31 (s, 3H, CH_3), 6.24 (d, J = 7.6 Hz, 1H, H5 Py), 8.04 (d, J = 7.6 Hz, 1H, H6 Py), 12.58 (bs, 1H, NH Py).

4-Methyl-2-oxo-1,2-dihydropyridine-3-carboxylic acid **(18)**

A mixture of 2-hydroxy-3-cyano-4-methylpyridine **17** (150.0 mg, 1.12 mmol) and 50% H_2SO_4 (0.2 mL) was stirred at 120 °C for 8 h. After, the reaction mixture was cooled to room temperature and few drops of water were added at 0 °C. The precipitate formed was filtered under vacuum, washed with water and dried to afford compound **18** (83.0 mg, 0.54 mmol) as a white solid.

Yield: 48%

^{1}H-NMR: (DMSO, 400 MHz) δ (ppm) 2.41 (s, 3H, CH_3), 6.57 (d, J = 7.6 Hz, 1H, H5 Py), 8.30 (d, J = 7.6 Hz, 1H, H6 Py), 13.33 (bs, 1H, COOH).

Methyl 4-methyl-2-oxo-1,2-dihydropyridine-3-carboxylate (19)

Method I:

A mixture of 4-methyl-2-oxo-1,2-dihydropyridine-3-carboxylic acid **18** (120.0 mg, 0.78 mmol), MeOH (0.5 mL) and concentrated H_2SO_4 (0.2 mL) was heated in a microwave reactor (CEM) at 100° C for 50 minutes (power = 200 W, pressure = 100 psi). After cooling the mixture was neutralized with solid Na_2CO_3 (pH 7-8) and extracted with CH_2Cl_2. The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure to give compound **19** (83.3 mg, 0.50 mmol) as a white solid.

Method II:

4-methyl-2-oxo-1,2-dihydropyridine-3-carboxylic acid **18** (475.0 mg, 3.10 mmol) was dissolved in MeOH (6.2 mL). Then the mixture was cooled to 0 °C and concentrated H_2SO_4 (3.1 mL) was added slowly. The reaction was refluxed at 85 °C overnight. After, MeOH was removed by evaporation under reduced pressure and solid Na_2CO_3 was added until a pH of 7-8 was reached. The mixture was then extracted with CH_2Cl_2, dried over anhydrous Na_2SO_4, filtered and evaporated under vacuum to afford compounds **19** (409.3 mg, 2.47 mmol) as a white solid.

Yield: 80%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 2.48 (s, 3H, CH_3), 3.90 (s, 3H, $COOCH_3$), 6.28 (d, *J* = 7.6 Hz, 1H, H5 Py), 8.20 (d, *J* = 7.6 Hz, 1H, H6 Py).

N-Cycloheptyl-4-Methyl-2-oxo-1,2-dihydropyridine-3-carboxamide (20)

Method I:

Methyl 4-Methyl-2-oxo-1,2-dihydropyridine-3-carboxylate **19** (187.0 mg, 1.13 mmol) was suspended in cyclohepthylamine (0.5 mL, 3.67 mmol) was heated in a microwave reactor (CEM) at 130° C for 30 minutes (power = 140 W, pressure = 100 psi). After cooling to room temperature, the reaction mixture was kept in an ice-water bath and treated with 10% HCl until a pH of 1-2 was reached. The precipitated formed was collected by filtration, washed with water and dried under vacuum to afford compound **20** (80.0 mg, 0.32 mmol) as a white solid.

Method II:

A suspension of methyl 4-Methyl-2-oxo-1,2-dihydropyridine-3-carboxylate **19** (964.0 mg, 5.81 mmol) in cyclohepthylamine (2.2 mL, 17.49 mmol) was refluxed at 150° C for 48 h. The mixture was cooled in an ice-water bath and 10% HCl was added until to pH 1-2. After that the white solid compound was collected by filtration, washed with water and dried under vacuum to give 440.0 mg of compound **20** (1.78 mmol).

Yield: 31%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 1.51-1.98 (m, 12H, cycloheptyl), 2,45 (s, 3H, CH_3), 4.17-4.27 (m, 1H, NH-CH cycloheptyl), 6.36 (d, *J* = 7.6 Hz, 1H, H5 Py), 8.55 (d, *J* = 7.6 Hz, 1H, H6 Py), 9.63 (bd, 1H, CONH), 12.96 (bs, 1H, NH Py) .

N-cycloheptyl-1-(4-fluorobenzyl)-4-methyl-2-oxo-1,2-dihydropyridine-3-carboxamide (1) and N-cycloheptyl-2-((4-fluorobenzyl)oxy)-4-methylnicotinamide (2)

N-Cycloheptyl-4-methyl-2-oxo-1,2-dihydropyridine-3-carboxamide **20** (75.0 mg, 0.30 mmol) was solubilized in anhydrous DMF (0.9 mL) and after CsF (138.5 mg, 0.91 mmol) was added. The mixture was stirred at room temperature for 1 h. Then, *p*-fluorobenzyl chloride (0.1 mL, 0.91 mmol) was added and the mixture obtained was stirred at 25 °C overnight. The reaction mixture was treated with ice-water and then repeatedly extracted with CH_2Cl_2. The combined organic layers were dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give a crude residue, which was purified by flash column chromatography on silica gel using, as eluent, a gradient mixture of petroleum ether/ethyl acetate from 8:2 to 5:5, affording compound **1** (57.2 mg, 0.16 mmol) as a brown oil and compound **2** (21.6 mg, 0.06 mmol) as a white-yellow solid.

Yield (**1**): 53%

^{1}H-NMR (**1**): ($CDCl_3$, 400 MHz) δ (ppm) 1.48 - 2.03 (m, 12H, cycloheptyl), 2.35 (s, 3H, CH_3), 4.12-4.13 (m, 1H, NH-CH cycloheptyl), 5.35 (s, 2H, N-CH_2), 6.30 (d, *J* = 7.6 Hz, 1H, H5 Py), 6.99-7.11 (m, 4H, ArH), 8.45 (d, *J* = 7.6 Hz, 1H, H6 Py), 9.72 (bd, 1H, CONH).

^{13}C-NMR (**1**): ($CDCl_3$, 100 MHz) δ (ppm) 21.42, 24.56, 28.44, 35.27, 47.40, 50.72, 116.17, 116.39, 119.36, 128.33, 131.48, 143.35, 150.59, 161.29, 163.44, 163.74.

Yield (**2**): 20%

^{1}H-NMR (**2**): ($CDCl_3$, 400 MHz) δ (ppm) 1.40-1.63 (m, 12H, cycloheptyl), 2.50 (s, 3H, CH_3), 4.10-4.20 (m, 1H, NH-CH cycloheptyl), 5.43 (s, 2H, O-CH_2), 6.91 (d, *J* = 8.0 Hz, 1H, H5 Py), 7.08-7.48 (m, 4H, ArH), 8.40 (d, *J* = 8.0 Hz, 1H, H6 Py).

5-Bromo-N-cycloheptyl-4-methyl-2-oxo-1,2-dihydropyridine-3-carboxamide (21)

N-cycloheptyl-4-methyl-2-oxo-1,2-dihydropyridine-3-carboxamide **20** (440.0 mg, 1.78 mmol) was dissolved in $CHCl_3$ (3.0 mL). Then a solution of Br_2 (0.1 mL, 1.78 mmol) in $CHCl_3$ (1.8 mL) was added dropwise to the mixture. The solution was stirred at room temperature overnight.

After the mixture was diluted with $CHCl_3$ and washed two times with saturated solution of $Na_2S_2O_3$. Then the organic phase was dried over anhydrous Na_2SO_4, filtered and evaporated under reduce pressure. The brown solid obtained was triturated with high boiling point petroleum ether to afford pure compound **21** (573.0 mg, 1.75 mmol).

Yield: 99%

^{1} H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 1.61 (m, 12H, cycloheptyl), 2.53 (s, 3H, CH_3), 4.19 (m, 1H, NH-CH cycloheptyl), 8.65 (s, 1H, H6 Py), 9.45 (bd, 1H, CONH), 13.40 (bs, 1H, NH Py).

5-*Bromo-N-cycloheptyl-1-(4-fluorobenzyl)-4-methyl-2-oxo-1,2-dihydropyridine-3-carboxamide (3) and 5-bromo-N-cycloheptyl-2-((4-fluorobenzyl)oxy)-4-methyl-2-oxo-1,2-dihydropyridine-3-carboxamide* **(4)**

5-bromo-*N*-cycloheptyl-4-methyl-2-oxo-1,2-dihydropyridine-3-carboxamide **21** was dissolved in anhydrous DMF (5.5 mL) and then CsF (278.8 mg, 1.84 mmol) was added. The mixture was stirred at room temperature for 1 h. After *p*-fluorobenzyl chloride (0.2 mL, 1.84 mmol) was added to the stirred suspension and the reaction was left under stirring at 25 °C, overnight.

The reaction was treated with ice-water and then repeatedly extracted with CH_2Cl_2. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give a crude residue that was purified by flash column chromatography on silica gel using the mixture petroleum ether/ethyl acetate 9:1 as eluent, affording the compound **3** (84.9 mg, 0.20 mmol) as a brown oil and compound **4** (108.0 mg, 0.25 mmol) as a white-brown solid.

Yield (**3**): 32%

^{1}H-NMR (3): ($CDCl_3$, 400 MHz) δ (ppm) 1.51 - 1.68 (m, 12H, cycloheptyl), 2.53 (s, 3H, CH_3), 4.12-4.17 (m, 1H, NH-CH cycloheptyl), 5.43 (s, 2H, N-CH_2), 7.03 -7.13 (m, 4H, ArH), 8.66 (s, 1H, H6 Py), 9.61 (bd, 1H, CONH);

^{13}C-NMR (3): ($CDCl_3$, 100 MHz) δ 21.15, 24.50, 28.44, 31.28, 49.02, 50.85, 102.18, 116.57, 128.27, 128.35, 130.96, 130.99, 161.40, 162.40, 163.85.

Yield (**4**): 41%

^{1}H-NMR (**4**): ($CDCl_3$, 400 MHz) δ (ppm) 1.37-1.86 (m, 12H, cycloeptyl), 2.61 (s, 3H, CH_3), 4.10-4.15 (m, 1H, NH-CH cycloheptyl), 5.42 (s, 2H, O-CH_2), 7.09-7.48 (m, 4H, ArH), 8.57 (s, 1H, H6 Py).

N-Cycloheptyl-1-(4-fluorobenzyl)-4-methyl-2-oxo-5-phenyl-1,2-dihydropyridine-3-carboxamide (5)

Dry toluene (1.7 mL) was placed in a vial together with PPh_3 (6.7 mg, 0.0255 mmol) and $Pd(OAc)_2$ (1.2 mg, 0,0051 mmol). Then the mixture was stirred at room temperature for 15 minutes to allow the formation of the catalyst tetrakis (triphenylphosphine)-palladium(0). After 5-bromo-*N*-cycloheptyl-1-(4-fluorobenzyl)-2-oxo-4-methyl-1,2-dihydropyridine-3-carboxamide **3** (75.0 mg, 0.17 mmol), anhydrous K_2CO_3 (35.9 mg, 0.26 mmol) and phenyl boronic acid (42.5 mg, 0.34 mmol) were added to the mixture. The reaction was heated at 100 °C under stirring, overnight.

After the reaction mixture was purified by flash column chromatography on silica gel, using a mixture of petroleum ether/ ethyl acetate (7:3) as eluent solvent. The proper fractions were collected and evaporated affording pure compound **5** (87.1 mg, 0.20 mmol) as a yellow oil.

Yield: > 99%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 1.53-1.69 (m, 12H, cycloheptyl), 2.30 (s, 3H, CH_3), 4.11-4.13 (m, 1H, NH-CH cycloheptyl), 5.46 (s, 2H, N-CH_2), 7.04-7.42 (m, 9H, ArH), 8.51 (s, 1H, H6 Py).

^{13}C-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 14.53, 24.52, 28.47, 31.27, 50.66, 50.75, 116.24, 122.29, 128.23, 128.97, 132.39, 161.30, 163.75, 171.51.

N-Cycloheptyl-1-(4-fluorobenzyl)-5-(4-methoxyphenyl)-4-methyl-2-oxo-1,2-dihydropyridine-3-carboxamide **(6)**

Dry toluene (1.2 mL) was placed in a vial together with PPh_3 (5.0 mg, 0.019 mmol) and $Pd(OAc)_2$ (0.9 mg, 0,004 mmol). Then the mixture was stirred at room temperature for 15 minutes to allow the formation of the catalyst tetrakis (triphenylphosphine)-palladium(0). After 5-bromo-*N*-cycloheptyl-1-(4-fluorobenzyl)-2-oxo-4-methyl-1,2-dihydropyridine-3-carboxamide **3** (54.8 mg, 0.13 mmol), anhydrous K_2CO_3 (26.1 mg, 0.19 mmol) and *p*-methoxyphenyl boronic acid (38.3 mg, 0.25 mmol) were added to the mixture. The reaction was heated at 100 °C under stirring, overnight.

After the reaction mixture was purified by flash chromatography using Buchi® pre-packed silica cartridges (mass = 4 g, particle size = 40-63 μm, flow = 20 mL/min) and a mixture of ethyl acetate/petroleum ether (2:8) as eluent solvent, affording 32.5 mg of compound **6** (0.07 mmol) as yellow-brown oil.

Yield: 54%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 1.53-1.67 (m, 12H, cycloheptyl), 2.17 (s, 3H, OCH_3), 2.30 (s, 3H, CH_3), 4.11-4.13 (m, 1H, NH-CH cycloheptyl), 5.40 (s, 2H, N-CH_2), 7.01-7.459 (m, 9H, ArH), 8.50 (s, 1H, H6 Py).

(E)-3-Oxo-2-(1-phenylethylidene) pentanenitrile (25)

Commercial available acetophenone **22** (1.2 mL, 10.05 mmol) and ethyl cyanoacetate **24** (1.3 mL, 12.06 mmol) were dissolved in dry CH_2Cl_2 (40.0 mL) and cooled to 0 °C in an ice bath. Neat $TiCl_4$ (2.2 mL, 20.10 mmol) was added dropwise to the solution. After this addition, the mixture was stirred for 30 minutes, and then dry pyridine (0.7 mL) was added dropwise always at 0 °C. The ice bath was subsequently removed and the solution was stirred at room temperature for 1 h. A further aliquot of dry pyridine (2.0 mL) was added dropwise and the mixture was stirred at room temperature overnight. 3 M HCl (40.0 mL) was added to the mixture and the organic layer separated. The acqueous layer was further extracted with CH_2Cl_2 (2 x 10.0 mL) and the combinated organics were washed with water and finally brine, and after dried over anhydrous Na_2SO_4, filtered and concentrated to give compound **25** (2.3 g, 10.60 mmol) as a viscous amber oil, which was used for the next step without further purification.

Yield: > 99%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 1.38 (t, *J* = 7.2 Hz, 3H, $OCH_2\mathit{CH_3}$), 2.70 (s, 3H, CH_3), 4.34 (q, *J* = 14.4 Hz, 2H, $O\mathit{CH_2}CH_3$), 7.43-7.47 (m, 5H, ArH).

(E)-2-(1-(4-Methoxyphenyl)ethylidene)-3-oxo-pentanenitrile **(26)**

Commercial available *p*-methoxyacetophenone **23** (1.5 g, 10.05 mmol) and ethyl cyanoacetate **24** (1.3 mL, 12.06 mmol) were dissolved in dry CH_2Cl_2 (40.0 mL) and cooled to 0°C in an ice bath. Neat $TiCl_4$ (2.2 mL, 20.10 mmol) was added dropwise to the solution. After this addition, the mixture was stirred for 30 minutes, and then dry pyridine (0.7 mL) was added dropwise always at 0 °C. The ice bath was subsequently removed and the solution was stirred at room temperature for 1 h. A further aliquot of dry pyridine (2.0 mL) was added dropwise and the mixture was stirred at room temperature overnight. 3 M HCl (40.0 mL) was added to the mixture and the organic layer separated. The acqueous layer was further extracted with CH_2Cl_2 (2 x 10.0 mL) and the combinated organics were washed with water and finally with brine, and after dried over anhydrous Na_2SO_4, filtered and concentrated to give compound **26** (5.3 g, 21.71 mmol) as a viscous amber oil, which was used for the next step without further purification.

Yield: > 99%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 1.38 (t, *J* = 7.2 Hz, 3H, $OCH_2\mathit{CH_3}$), 3.83 (s, 3H, CH_3), 3.85 (s, 3H, OCH_3), 4.33 (q, *J* = 7.2 Hz, 2H, $O\mathit{CH_2}CH_3$), 6.95-7.48 (m, 4H ArH).

(*2E,4E)-5-(Dimethylamino)-3-phenyl-2-propionylpenta-2,4-dienenitrile* (**27**)

A mixture of (*E*)-3-oxo-2-(1-phenylethylidene)pentanenitrile **25** (1.0 g, 4.65 mmol) and *N,N*-dimethylformamide-dimethyl acetal (0.6 mL, 4.65 mmol) was stirred at room temperature without any solvent for 24 h. The purple solid obtained was triturated several times with diethyl ether and, at last, the solid was filtered under vacuum to afford compound **27** (835.5 mg, 3.09 mmol) as a yellow solid, which was used for the next step without further purification.

Yield: 67%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 1.34 (t, *J* = 7.2 Hz, 3H, $OCH_2\mathit{CH_3}$), 3.00 (s, 6H, $N(CH_3)_2$), 4.26 (q, *J* = 7.2 Hz, 2H, $O\mathit{CH_2}CH_3$), 6.51 (d, *J* = 12.8 Hz, 1H, CH=*CH*), 7.21 (d, *J* = 12.8 Hz, 1H, *CH*=CH), 7.24-7.41 (m, 5H, ArH).

(2E,4E)-5-(Dimethylamino)-3-(4-methoxyphenyl)-2-propionylpenta-2,4-dienenitrile (28)

A mixture of (*E*)-2-(1-(4-methoxyphenyl)ethylidene)-3-oxo-pentanenitrile **26** (2.7 g, 10.85 mmol) and *N,N*-dimethylformamide-dimethyl acetal (1.4 mL, 10.85 mmol) was stirred at room temperature without any solvent for 24 h. The purple solid obtained was triturated several times with diethyl ether and then filtered under vacuum affording compound **28** (1.7 g, 5.82 mmol) as a yellow-green solid, which was used for the next step without further purification.

Yield: 54%

^{1}H-NMR: (DMSO, 400 MHz) δ (ppm) 1.26 (t, *J* = 7.2 Hz, 3H, OCH_2CH_3), 3.01 (s, 3H, $N\text{-}CH_3$), 3.08 (s, 3H, $N\text{-}CH_3$), 3.85 (s, 3H, OCH_3), 4.17 (q, *J* = 6.8 Hz, 2H, OCH_2CH_3), 6.75 (d, *J* = 12.4 Hz, 1H, CH=*CH*), 7.12 (d, *J* = 12.8 Hz, 1H, *CH*=CH), 7.02-7.20 (m, 4H, ArH).

1-(4-Fluorobenzyl)-2-oxo-4-phenyl-1,2-dihydropyridine-3-carbonitrile (29)

A mixture of (2*E*,4*E*)-5-(dimethylamino)-3-phenyl-2-propionylpenta-2,4-dienenitrile **27** (271.5 mg, 1.00 mmol) and *p*-flurobenzylamine (0.1 mL, 1.00 mmol) was refluxed at 90 °C for 2 h. After cooling, the solid obtained was triturated several times with diethyl ether to give compound **29** (251.0 mg, 0.82 mmol) as a brown solid.

Yield: 82%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 2.55 (s, 2H, N-CH_2), 6.63 (d, *J* = 6.8 Hz, 1H, H5 Py), 7.23-7.71 (m, 9H, ArH), 8.35 (d, *J* = 7.2 Hz, 1H, H6 Py).

1-(4-Fluorobenzyl)-4-(4-methoxyphenyl)-2-oxo-1,2-dihydropyridine-3-carbonitrile **(30)**

A mixture of (2*E*,4*E*)-5-(dimethylamino)-3-(4-methoxyphenyl)-2-propionylpenta-2,4-dienenitrile **28** (144.8 mg, 0.48 mmol) and *p*-flurobenzylamine (0.1 mL, 0.48 mmol) was refluxed at 90 °C for 2 h. After cooling, the solid obtained was was triturated several times with diethyl ether to give compound **30** (125.0 mg, 0.37 mmol) as a brown solid.

Yield: 78%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 3.86 (s, 3H, OCH_3), 5.14 (s, 2H, N-CH_2), 6.32 (d, *J* = 6.8 Hz, 1H, H5 Py), 7.00-7.09 (m, 4H, ArH), 7.36 - 7.62 (m, 4H, ArH), 7.48 (d, *J* = 6.8 Hz, 1H, H6 Py).

Methyl-2-methoxynicotinate (32)

Sodium (296.0 mg, 12.88 mmol) was added to dried methanol (8.0 mL). After the dissolvation of sodium, commercial available methyl-2-chloro-pyridine-3-carboxylate (**31**) (1.5 mL, 11.66 mmol) was added and the mixture was refluxed at 65 °C for 6 h. Then water and ethyl acetate were added to the solution and the organic layer was separated. The water layer was extracted with ethyl acetate for three times. The organic phases were unified and dried over anhydrous Na_2SO_4, filtered and evaporated, affording compound **32** (1.6 g, 9.72 mmol) as a colorless oil that was used for the next step without further purification.

Yield: 83%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 3.90 (s, 3H, OCH_3), 4.04 (s, 3H, $COOCH_3$), 6.94 (m, 1H, H5 Py), 8.16 (dd, J_1 = 6 Hz, J_2 = 2 Hz, 1H, H6 Py), 8.31 (dd, J_1 = 5.2 Hz, J_2 = 2.0 Hz, 1H, H4 Py).

Methyl 1-(4-fluorobenzyl)-2-oxo-1,2-dihydropyridine-3-carboxylate **(33)**

Methyl 2-methoxynicotinate **32** (420.5 mg, 2.52 mmol) was dissolved in *p*-fluorobenzyl bromide (0.4 mL, 3.20 mmol) under heating and the solution was further heated at 120 °C for 20 h.

After, pure compound **33** (589.7 mg, 2.26 mmol) was obtained by purification by flash column chromatography of the final reaction mixture over silica gel and petroleum ether/ ethyl acetate (6:4) as eluent mixture.

Yield: 90%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 3.90 (s, 3H, OCH_3), 5.13 (s, 2H, N-CH_2), 6.24 (t, J = 7.2 Hz, 1H, H5 Py), 7.00-7.38 (m, 4H, ArH), 7.53 (dd, J_1 = 6.4 Hz, J_2 = 2 Hz, 1H, H6 Py), 8.15 (dd, J_1 = 7.2 Hz, J_2 = 2.4 Hz, 1H, H4 Py).

Methyl 1-(4-fluorobenzyl)-2-oxo-4-phenyl-1,2,3,4-tetrahydropyridine-3-carboxylate (34)

Methyl 1-(4-fluorobenzyl)-2-oxo-1,2-dihydropyridine-3-carboxylate **33** (294.0 mg, 1.13 mmol) was dissolved in dry THF (6.5 mL). Copper(I) iodide (41.9 mg, 0.22 mmol) and lithium bromide (19.1 mg, 0.22 mmol) were added under nitrogen atmosphere and the solution was cooled down to -40°C.

The phenylmagnesium bromide (1 M in THF, 1.8 mL, 1.82 mmol) was added dropwise, always under nitrogen. After additional 6 h the stirred solution was warmed up to room temperature. After a 20% solution of ammonium chloride (5.5 mL) and diethyl ether (6.9 mL) were added. The organic phase was separated and the water layer was extracted two times with diethyl ether (2 x 6.92 mL). The organic phases were unified and washed with 20% solution of ammonium chloride (13.8 mL), water (13.8 mL), twice with a 10% solution of hydrochloric acid (2 x 13.8 mL), water (13.8 mL) and brine. After the organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated, affording a mixture difficult to purify. Therefore compound **34** was not isolated.

1-(4-Fluorobenzyl)-2-oxo-1,2-dihydropyridine-3-carboxylic acid **(39)**

Commercial available 2-hydroxynicotinic acid **38** (1.5 g, 10.78 mmol) was dissolved in anhydrous DMF (20.0 mL) and then NaH (650.0 mg, 27.08 mmol) was slowly added. The mixture was left under stirring at room temperature for 1 h. After *p*-fluorobenzyl chloride (1.5 mL, 12.52 mmol) was added and the mixture was heated at 50 °C overnight.

Then, DMF was removed under reduced pressure affording a white solid that was triturated several times with water and then filtered under vacuum.

To the solid obtained from the previous step a solution of 10 % NaOH (20.0 mL) was added and the mixture was refluxed at 100 °C for 4 h. Then the mixture was acidified with concentrated HCl until a pH 1-2 was reached, and the precipitated formed was filtered under vacuum, washed with water and dried under vacuum to afford pure compound **39** (2.6 g, 10.36 mmol).

Yield: 96%

^{1}H-NMR: (DMSO, 400 MHz) δ (ppm) 5.34 (s, 2H, N-CH_2), 6.81 (t, J = 7.2 Hz, 1H, H5 Py), 7.22-7.50 (m, 4H, ArH), 8.42-8.47 (m, 2H, H4 and H6 Py).

N-Cycloheptyl-1-(4-fluorobenzyl)-2-oxo-1,2-dihydropyridine-3-carboxamide (40)

1-(4-fluorobenzyl)-2-oxo-1,2-dihydropyridine-3-carboxylic acid **39** (3.4 g, 13.76 mmol) was dissolved in dried DMF (40.0 mL). After DIPEA (7.2 mL, 41.28 mmol) and TBTU (4.4 g, 13.76 mmol) were added at 0 °C, under N_2, and the reaction was left at 0 °C for 30 minutes. After, cycloheptylamine (1.8 mL, 13.76 mmol) was added at 0 °C and the mixture was left at room temperature overnight. After evaporation of the solvent, the crude solid obtained was purified by flash column chromatography on silica gel using the mixture ethyl acetate/petroleum ether (6:4) as eluent. After evaporation of the opportune fraction, pure compound **40** (3.3 g, 9.70 mmol) as a brown oil.

Yield: 71%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 1.53-2.02 (m, 12H, cycloheptyl), 4.11-4.16 (m, 1H, NH-CH cycloheptyl), 5.18 (s, 2H, CH_2), 6.40 (t, J = 6.8 Hz, 1H, H5 Py), 7.03-7.29 (m, 4H, ArH), 7.47 (dd, J_1 = 6.8 Hz, J_2 = 2.4 Hz, 1H, H6 Py), 8.53 (dd, J_1 = 7.2 Hz, J_2 = 2.4 Hz, 1H, H4 Py).

N-Cycloheptyl-1-(4-fluorobenzyl)-2-oxo-4-phenyl-1,2-dihydropyridine-3-carboxamide (7)

N-Cycloheptyl-1-(4-fluorobenzyl)-2-oxo-1,2-dihydropyridine-3-carboxamide **40** (1.3 g, 3.86 mmol) was dissolved in dry THF (28.2 mL), and copper(I) iodide (143.4 mg, 0.75 mmol) was added under nitrogen atmosphere, followed by lithium bromide (65.4 mg, 0.75 mmol). After, the stirred solution was cooled to -40 °C and phenylmagnesium bromide (1 M in THF, 7.72 mL, 6.18 mmol) was added dropwise, always under nitrogen. The reaction mixture was then left under stirring at -40 °C After additional 6 h the stirred solution was warmed up to room temperature. After a 20% solution of ammonium chloride (19.0 mL) and diethyl ether (24 mL) were added. The organic phase was separated and the water layer was extracted two times with diethyl ether (2 x 24 mL). The organic phases was collected and washed with 20% solution of ammonium chloride (45 mL), water (45 mL), twice with a 10% solution of hydrochloric acid (2 x 45 mL), water (45 mL) and brine. After the organic layer was was dried over anhydrous Na_2SO_4, filtered and evaporated to afford a crude residue that was purified by flash column chromatography on silica gel, using a mixture of toluene/ ethyl acetate (3:7) as eluent solvent. The proper fractions were collected and evaporated, afforded compound **7** (1.5 g, 3.55 mmol) as a brown oil.

Yield: 92%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 1.25-1.89 (m, 12H, cycloheptyl), 3.97-3.99 (m, 1H, NH-CH cycloheptyl), 5.15 (s, 2H, N-CH_2), 6.23 (d, *J* = 7.2 Hz, 1H, H5 Py), 7.03-7.45 (m, 9H, ArH), 7.46 (d, *J* = 7.2 Hz, 1H, H6 Py).

N-Cycloheptyl-1-(4-fluorobenzyl)-4-(4-methoxyphenyl)-2-oxo-1,2-dihydropyridine-3-carboxamide (8)

N-Cycloheptyl-1-(4-fluorobenzyl)-2-oxo-1,2-dihydropyridine-3-carboxamide **40** (2.0 g, 5.84 mmol) was dissolved in dry THF, and copper(I) iodide (216,9 mg, 1.14 mmol) was added under nitrogen atmosphere, followed by lithium bromide (98.9 mg, 1.14 mmol). After, the stirred solution was cooled to -40 °C and *p*-methoxyphenylmagnesium bromide (0.5 M in THF, 19.7 mL, 9.34 mmol) was added dropwise, always under nitrogen. The reaction mixture was then left under stirring at -40 °C After additional 6 h the stirred solution was warmed up to room temperature. After a 20% solution of ammonium chloride (28.0 mL) and diethyl ether (36.0 mL) were added. The organic phase was separated and the water layer was extracted two times with diethyl ether (2*36.0 mL). The organic phases was collected and washed with 20% solution of ammonium chloride (69.0 mL), water (69.0 mL), twice with a 10% solution of hydrochloric acid (2*69.0 mL), water (69.0 mL) and brine. After the organic layer was was dried over anhydrous Na_2SO_4, filtered and evaporated to afford a crude residue that was purified by flash column chromatography on silica gel using a mixture of ethyl acetate/petroleum ether (7:3) as eluent solvent. The proper fractions were collected and evaporated, afforded compound **8** (352.1 mg, 0.79 mmol) as a brown oil.

Yield: 13%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 1.40-1.58 (m, 12H, cycloheptyl), 3.45-3.50 (m, 1H, NH-CH cycloheptyl), 3.82 (s, 3H, OCH_3), 5.14 (s, 2H, N-CH_2), 6.23 (d, J = 6.8 Hz, 1H, H5 Py), 6.89-7.07 (m, 4H, ArH), 7.29-7.35 (m, 5H, H6 Py and ArH).

5-Bromo-N-cycloheptyl-1-(4-fluorobenzyl)-2-oxo-4-phenyl-1,2-dihydropyridine-3-carboxamide (9)

N-Cycloheptyl-1-(4-fluorobenzyl)-2-oxo-4-phenyl-1,2-dihydropyridine-3-carboxamide **7** (191.0 mg, 0.46 mmol) was dissolved in $CHCl_3$ (0.8 mL). A solution of Br_2 (0.02 mL, 0.46 mmol) in $CHCl_3$ (0.5 mL) was then added dropwise and the mixture was left under stirring at room temperature overnight. After the mixture was diluted with $CHCl_3$ and washed two times with a saturated solution of $Na_2S_2O_3$. The organic layer was then dried over anhydrous Na_2SO_4, filtered and evaporated under reduce pressure. The brown solid obtained was triturated with high boiling point petroleum ether to afford pure compound **9** (142.1 mg, 0.29 mmol).

Yield: 63%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 1.36-1.74 (m, 12H, cycloheptyl), 3.87-3.89 (m, 1H, NH-CH cycloheptyl), 5.13 (s, 2H, N-CH_2), 7.06-7.43 (m, 9H, ArH), 7.60 (s, 1H, H6 Py).

5-*Bromo-N-cycloheptyl-1-(4-fluorobenzyl)-4-(4-methoxyphenyl)-2-oxo-1,2-dihydropyridine-3-carboxamide* (10)

N-Cycloheptyl-1-(4-fluorobenzyl)-4-(4-methoxyphenyl)--2-oxo-1,2-dihydropyridine-3-carboxamide **8** (315.7 mg, 0.70 mmol) was dissolved in $CHCl_3$ (1.2 mL). A solution of Br_2 (0.036 mL, 0.70 mmol) in $CHCl_3$ (0.7 mL) was then added dropwise and the mixture was left under stirring at room temperature overnight.

After the mixture was diluted with $CHCl_3$ and washed two times with a saturated solution of $Na_2S_2O_3$. The organic layer was then dried over anhydrous Na_2SO_4, filtered and evaporated under reduce pressure. The brown solid obtained was triturated with high boiling point petroleum ether to afford pure compound **10** (400.0 mg, 0.75 mmol).

Yield: > 99%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 1.21-1.54 (m, 12H, cycloheptyl), 3.81 (s, 3H, OCH_3), 3.82-3.98 (m, 1H, NH-CH cycloheptyl), 5.11 (s, 2H, N-CH_2), 6.91-7.40 (m, 8H, ArH), 7.59 (s, 1H, H6 Py).

N-Cycloheptyl-1-(4-fluorobenzyl)-2-oxo-4,5-diphenyl-1,2-dihydropyridine-3-carboxamide (11)

Dry toluene (2.5 mL) was placed in a vial together with PPh_3 (10.2 mg, 0.039 mmol) and $Pd(OAc)_2$ (1.9 mg, 0.0078 mmol). Then the mixture was stirred at room temperature for 15 minutes to allow the formation of the catalyst tetrakis(triphenylphosphine)-palladium(0). After, 5-bromo-*N*-cycloheptyl-1-(4-fluorobenzyl)-2-oxo-4-phenyl-1,2-dihydropyridine-3-carboxamide **9** (130.0 mg, 0.26 mmol), potassium carboxylate (53.9 mg, 0.39 mmol) and phenyl boronic acid (65.0 mg, 0.52 mmol) were added to the mixture. The vial was sealed and the solution was heated at 100 °C overnight.

After the reaction mixture was purified by flash column chromatography on silica gel using a mixture of ethyl acetate/petroleum ether (7:3) as eluent solvent, affording 117.1 mg of compound **11** (0.24 mmol) as brown oil.

Yield: 91%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 1.38-1.51 (m, 12H, cycloheptyl), 3.90-4.00 (m, 1H, NH-CH cycloheptyl), 5.22 (s, 2H, N-CH_2), 7.06-7.51 (m, 14H, ArH), 7.37 (s, 1H, H6 Py).

^{13}C-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 24.20, 28.37, 34.93, 50.58, 52.19, 116.14, 122.41, 127.07, 127.35, 128.33, 130.82, 130.90, 136.85, 151.95, 160.26, 164.29.

N-Cycloheptyl-1-(4-fluorobenzyl)-4-(4-methoxyphenyl)-2-oxo-5-phenyl-1,2-dihydropyridine-3-carboxamide (12)

Dry toluene (3.6 mL) was placed in a vial together with PPh_3 (14.5 mg, 0.0551 mmol) and $Pd(OAc)_2$ (2.7 mg, 0.011 mmol). Then the mixture was stirred at room temperature for 15 minutes to allow the formation of the catalyst tetrakis(triphenylphosphine)-palladium(0). After, 5-bromo-*N*-cycloheptyl-1-(4-fluorobenzyl)-2-oxo-4-(4-methoxyphenyl)-1,2-dihydropyridine-3-carboxamide **10** (195.0 mg, 0.37 mmol), potassium carboxylate (76.2 mg, 0.55 mmol) and phenyl boronic acid (91.7 mg, 0.73 mmol) were added to the mixture. The vial was sealed and the solution was heated at 100° C overnight.

After the reaction mixture was purified by flash column chromatography on silica gel using a mixture of ethyl acetate/petroleum ether (8:2) as eluent solvent, affording 100.0 mg of compound **12** (0.19 mmol) as brown oil.

Yield: 51%

^{1}H-NMR: : ($CDCl_3$, 400 MHz) δ (ppm) 1.39-1.64 (m, 12H, cycloheptyl), 3.50-3.59 (m, 1H, NH-CH cycloheptyl), 3.83 (s, 3H, OCH_3), 4.99 (s, 2H, N-CH_2), 6.94-7.40 (m, 13H, ArH), 8.03 (s, 1H, CONH), 8.76 (s, 1H, H6 Py).

N-Cycloheptyl-1-(4-fluorobenzyl)-5-(4-methoxyphenyl)-2-oxo-4-phenyl-1,2-dihydropyridine-3-carboxamide **(13)**

Dry toluene (3.9 mL) was placed in a vial together with PPh_3 (15.8 mg, 0.0603 mmol) and $Pd(OAc)_2$ (3.0 mg, 0.012 mmol). Then the mixture was stirred at room temperature for 15 minutes to allow the formation of the catalyst tetrakis(triphenylphosphine)-palladium(0). After, 5-bromo-*N*-cycloheptyl-1-(4-fluorobenzyl)-2-oxo-4-phenyl-1,2-dihydropyridine-3-carboxamide **9** (200.0 mg, 0.40 mmol), potassium carboxylate (83.3 mg, 0.60 mmol) and *p*-methoxyphenyl boronic acid (122.2 mg, 0.80 mmol) were added to the mixture. The vial was sealed and the solution was heated at 100° C overnight.

After the reaction mixture was purified by flash chromatography using Buchi® pre-packed silica cartridges (mass = 12 g, particle size = 40-63 μm, flow = 30 mL/min) and a mixture of ethyl acetate/petroleum ether (6:4) as eluent solvent, affording 144.2 mg of compound **13** (0.28 mmol) as yellow-brown oil.

Yield: 70%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 1.32-1.51 (m, 12H, cycloheptyl), 3.70 (s, 3H, OCH_3), 3.85-3.90 (m, 1H, NH-CH cycloheptyl), 5.17 (s, 2H, N-CH_2), 6.62-7.39 (m, 13H, ArH), 7.31 (s, 1H, H6 Py).

N-Cycloheptyl-1-(4-fluorobenzyl)-4,5-bis(4-methoxyphenyl)-2-oxo-1,2-dihydropyridine-3-carboxamide **(14)**

Dry toluene (3.6 mL) was placed in a vial together with PPh_3 (14.5 mg, 0.0551 mmol) and $Pd(OAc)_2$ (2.7 mg, 0.011 mmol). Then the mixture was stirred at room temperature for 15 minutes to allow the formation of the catalyst tetrakis(triphenylphosphine)-palladium(0). After, 5-bromo-*N*-cycloheptyl-1-(4-fluorobenzyl)-2-oxo-4-(4-methoxyphenyl)-1,2-dihydropyridine-3-carboxamide **10** (195.0 mg, 0.37 mmol), potassium carboxylate (76.2 mg, 0.55 mmol) and *p*-methoxyphenyl boronic acid (111.5 mg, 0.73 mmol) were added to the mixture. The vial was sealed and the solution was heated at 100° C overnight.

After the reaction mixture was purified by flash chromatography using Buchi® pre-packed silica cartridges (mass = 4 g, particle size = 40-63 μm, flow = 20 mL/min) and a mixture of ethyl acetate/petroleum ether (6:4) as eluent solvent, affording 73.0 mg of compound **14** (0.13 mmol) as yellow-brown oil.

Yield: 35%

^{1}H-NMR: ($CDCl_3$, 400 MHz) δ (ppm) 1.39-1.56 (m, 12H, cycloheptyl), 2.05 (s, 6H, OCH_3), 3.90-3.93 (m, 1H, NH-CH cycloheptyl), 5.16 (s, 2H, N-CH_2), 6.65-7.05 (m, 12H, ArH), 7.37 (s, 1H, H6 Py).

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Infine, lo dedico a me e concludo con una citazione…

Schopenahauer diceva: "La vita e i sogni sono le pagine di uno stesso libro: leggerle in ordine è vivere, sfogliarle a caso è sognare.", adesso credo proprio che una di quelle pagine sfogliate a caso si sia realizzata!