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NEW PLAYERS IN AN OLD GAME:

Pharmacological Evaluation of the Benzomorphan-Based Compound LP1

Design and Synthesis of Conformationally Constrained Compounds as New Tramadol-like Candidates

PhD THESIS

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INDEX OF CONTENTS

ABSTRACT

For the clinical management of acute and chronic pain, a possible therapeutic approach is the use of associations between two or more drugs, which produce their biological effects on two or more different sites of action, in order to modulate directly or indirectly profile analgesic and adverse effects (Argoff 2011). Given the advantages of polypharmacology, in the drug discovery process has been established the strategy "one-molecule multiple targets" (Morphy and Rankovic, 2009). The multitarget ligands provided better analgesic activity and fewer side effects - already observed in the association of drugs – coupled with favourable pharmacokinetic and pharmacodynamic characteristics. It is known that endogenous opioid peptides are the key mediators in the modulation of the pain transmission in descending pathways. Similarly, monoamine neurotransmitters - according to the location and type of receptor involved - can positively or negatively modulate the transmission of pain sensation. Considering that the various mediators involved in the circuit of pain represent potential targets for different pharmacological interventions, multitarget ligands possessing opioid-opioid or non-opioid-opioid mechanisms of action are potential drug candidates for the management of various pain conditions.

Multitarget ligands, able to act simultaneously on multiple opioid receptors subtypes, showed low propensity to induce side effects. In particular, it was found an improved analgesic profile associated with a reduced tendency to induce tolerance in mixed MOR (mu-opioid receptor)-

DOR (delta-opioid receptor) ligands (Schiller 2010). In previous studies was demonstrated that the benzomorphan-based compound LP1 (Pasquinucci et al. 2010) exhibited high and good affinities *versus* MOR and DOR, respectively, and an analgesic potency comparable to morphine completely NX-reversed. Given this background, the present thesis focused on the study of the functional profile of LP1 through [^{35}S]GTPγS binding assay and tail flick test using selective MOR, DOR and KOR antagonists. Moreover, to further delineate its pharmacological profile, NX-M was administered either s.c. or i.v. to investigate if the LP1 action is centrally- or peripherally-mediated and it was also measured the LP1 ability to induce tolerance in regimen of repeated administration. Finally, LP1's behavioural effects – antihyperalgesia and antiallodynia – in animal models of persistent pain were studied. Collected data indicated LP1 as a central-acting MOR agonist-DOR antagonist (Parenti et al., 2012b) with low capability to induce tolerance (Pasquinucci et al., 2012). Moreover, antihyperalgesic and antiallodynic effects of LP1 in animal model of persistent pain suggested this multitarget compound as a possible useful tool for chronic pain treatment.

In the multitarget ligand context, another strategy widely investigated is the combination of the MOR agonism with the monoamines reuptake inhibition (Bannister et al. 2009). A compound corresponding to this pharmacological profile is Tramadol (Leppert 2009), which has been selected as lead compound. The chemistry program was engaged with the aim to obtain compounds that maintained the mechanism of action of the

parental ligand and showed an improved analgesic efficacy. First, pharmacophoric features and their critical distances have been highlighted to identify a model that represented the interaction with MOR of Tramadol and its derivatives. Then, it has been designed and synthesised a series of conformationally constrained compounds as new Tramadol-like candidates, in which two pharmacophoric elements of tramadol - the lateral chain and the basic nitrogen - are constrained in a cyclised structures represented by the *trans*-decahydroisoquinoline and octahydro-1*H*-cyclopenta[c]pyridine nuclei. [*]

[*] **ABBREVIATIONS**: MOR, μ-opioid receptor; DOR, δ-opioid receptor; KOR, κ-opioid receptor; LP1, 3-[(2*R*,6*R*,11*R*)-8-hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2*H*)-yl]-*N*-phenylpropanamide; Tramadol HCl, (1*RS*,2*RS*)-2-[(dimethyl-amino)methyl]-1-(3-methoxyphenyl)-cyclohexanol; s.c., subcutaneous; i.v., intracerebroventricular; NX, naloxone; NX-M, naloxone methiodide.

Chapter 1

INTRODUCTION

Pain

Pain, as defined by the International Association for the Study of Pain (IASP), is an "*unpleasant sensory and emotional experience associated with actual or potential tissue damage*" (IASP 1994; Fishbain et al. 2010).

Pain is characterised by perceptive and emotional components. The first component is the **nociception** by which the perception of a negative and detrimental stimulus for the organism is transmitted to the CNS. The **emotional** component, entirely beholder, represents the psychic perception of pain that can be more or less intense as function of the emotional state of the individual. Thus, the pain experience is the result of the affective and cognitive dimensions, strongly influenced by psychic structure and socio-cultural factors.

Pain is physiological when it is an alarm signal for a tissue injury representing a system of defence essential to avoid damage. Pain becomes pathological when it remains, losing the initial meaning and becoming a disease (Mannion and Woolf 2000).

Pain is also defined and classified on the basis of either its persistence over time and underlying physical and psychological causes. As reported by Mannion and Woolf (2000) a first distinction, based on the pain persistence over time, is made between acute physiological pain and chronic pathological pain. **Acute pain** is a protective mechanism triggered by a specific physical cause and resolves in a short time following causal resolution. In contrast, **chronic pain** is commonly defined as persistent

pain and lasts beyond the ordinary duration of time that an injury needs to heal.

Considering causal factors, pain is commonly designated as nociceptive, neuropathic, inflammatory, functional, somatoform, or existential (Fig. 1.1). While acute pain is mainly caused by nociceptive stimuli - thermal, mechanical or chemical – chronic pain can be produced via nociceptive, neuropathic, existential, or mixed stimuli.

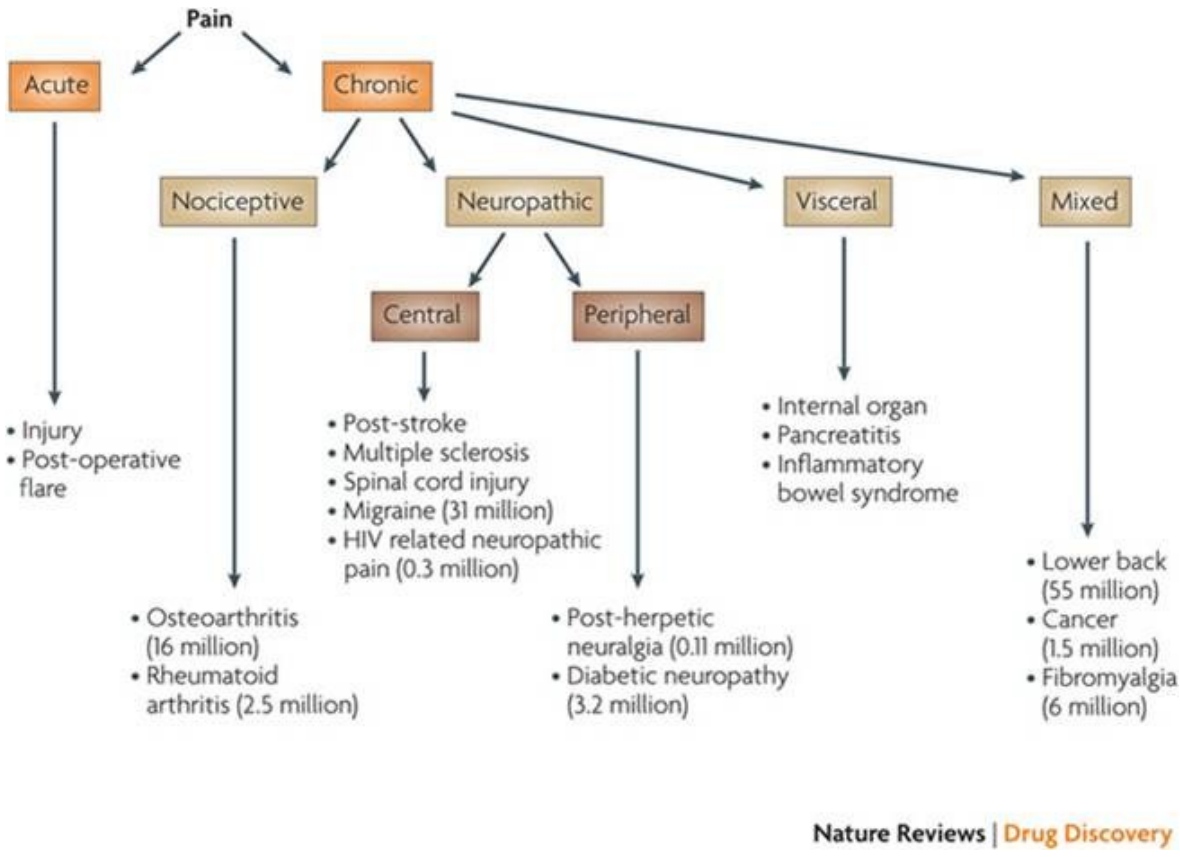


Figure 1.1. Pain Classification.

Neuropathic pain, due to neuronal lesions or nervous system dysfunctions, can be the consequence of an ectopic neuronal firing, or the result of releasing peptides by injured nerve contributing to inflammatory response, or it can be caused by inhibition of pathways in the brain and

spinal cord involved in transmitting peripheral signals. Inflammatory pain refers to spontaneous pain and hypersensitivity due to tissue damage or inflammation. Sensory afferent nerves are sensitive to inflammation and its chemical mediators, including bradykinins, prostaglandins, and leukotrienes. The stimulation of afferent neurons by inflammatory mediators can cause peripheral and central sensitization and a change in neuronal function that may evolve in chronic pain. However, a clear distinction between chronic neuropathic pain and chronic inflammatory pain is no always possible. In fact, typical components of an inflammatory process may become part of the mechanisms regulating neuropathic pain, since inflammation can trigger nerve injury and in this way produce neuropathic pain. Similarly, nerve injuries can lead to an inflammatory reaction (neurogenic inflammation) that contributes to the inflammatory pain expression.

Functional pain (non-nociceptive or non-neuropathic) probably due to abnormal pain processing or functioning of the nervous system, resulting in allodynia - from a stimulus that does not normally provoke an algesic response - and hyperalgesia - which is an increased response to a stimulation that is normally painful.

Somatoform pain is characterised by a strong psychological component. In this condition, patients complain of pain in one or more areas without the presence of a detectable medical cause. This form of pain is often accompanied by anxiety manifestations and often has effects on the patient's social life (Christo and Mazloomdoost, 2008; Omoigui 2007).

Pain pathways

Ascending pathway

Painful feelings originate from peripheral nociceptors (Omoigui 2007). In contrast to other types of sensory fibres, such as those for the sense of touch having at their endings specialized structures (such as Pacinian and Messner corpuscles), nociceptive fibres (the fibres carrying pain signals) possess free nerve endings. These free nerve endings form dense networks that are regarded as nociceptors. They are high threshold receptors, activated by stimuli of sufficient intensity to cause tissue damage.

Substance P, bradykinin, serotonin and histamine are endogenous substances typically realised in tissue lesions and capable to stimulate the peripheral nociceptors.

Nociceptors (Caterina et al. 2005) are distinguished in mechanical, activated by very intense stimulation (as sharp objects), thermal nociceptors, activated by temperatures above 45 °C, and polymodal nociceptors, activated by all types of stimuli. There are various types of nerve fibres (axons) whose free endings form nociceptors. These fibres connect all peripheral organs to the spinal cord and differ greatly both in diameter and in the thickness of the myelin sheath that surrounds them.

Primary afferent fibres are classified according to three physiological criteria: 1) the speed of conduction, 2) the type of stimulus that evokes the response and 3) the temporal characteristics of the response to the stimulus. On the basis of these criteria, primary afferent fibres are distinguished in C and Aδ fibres. C fibres, unmyelinated and to slow speed of conduction, respond to mechanical and thermal stimuli. Aδ fibres

are myelinated and to faster conduction. There are three distinct types of Aδ fibres. Type I and type II Aδ fibres typically respond to heat, mechanical and chemical stimuli. Another type of Aδ, unresponsiveness to thermal stimuli, has been named high-threshold mechanoreceptors of large calibre, myelinated and to rapid conduction.

Painful stimuli cause the opening of ion channels and then flux of ions across cell membranes within the peripheral nociceptive afferent (Woolf 2004). Sufficiently strong stimuli generate depolarization and potential of action that are conducted via peripheral afferents to the dorsal horn of the spinal cord (Bingham et al. 2009).

After transmission to the second order neuron, the pain stimulus is transmitted to various supra-spinal structures via afferent fibres.

The cellular bodies of the afferent fibres are located in the spinal dorsal root ganglia. They enter the spinal cord through the dorsal roots and terminate in the grey matter of the posterior horns. C fibres project in the superficial layers of laminae I and II, whereas the Aδ fibres innervate the cell bodies of the lamina V. From cellular body of neurons in the laminae I and V originate the ascendant fibres projecting in the thalamus constituting the ipsilateral and contralateral spino-thalamic beams, which innervate the somato-sensory cortex. The somato-sensorial information is transmitted to the cerebral cortex through two ascendant systems, a lateral and a medial systems. The spino-thalamic tract is part of the lateral system and project to the thalamus. It elaborates the sensory and discriminative aspects of pain information. The spino-reticular-thalamic tract is part of the medial system and project to the reticular formation of

the brainstem and to the thalamus. Thalamus is a symmetric part of the brain and constitutes the main part of the diencephalon. The thalamus acts as a relay station, which disseminate the signals to various areas of the brain, including the cerebral cortex.

Cerebral cortex is the part of the brain where the perception as pain takes place. The perception of pain is due to the nociceptive transmission from the thalamus to the sensory cortex by the thalamus-cortical neuronal fibres. The processing of pain perception also involves structures pre-frontal, frontal and limbic (amygdala and hippocampus). Limbic system is a regulation centre of the pain threshold and of emotional reactions, thus the perception conscious arising from peripheral sensory input overlap the cognitive and affective components.

Cells of laminae II and III of the dorsal horn (SG) are inhibitory interneurons that innervate the laminae I and V. Interneurons are rich of opioid peptides and opioid receptors that activated by inhibitory descending pathway regulate the transmission between the primary afferent fibres and spino-thalamic tract neurons.

Descending pathway

Descending pathway modulates pain sensory. An important area for the descending pain modulation is the PAG. The PAG, an area rich of opioid peptides, receives inputs from different brain areas such as hypothalamus, cortex and thalamus. From PAG originate fibres that innervate an area of the brainstem called the RMN rich in serotonergic neurons. Hence, the fibres run in the dorsolateral funiculus of the spinal cord and terminate forming synapses with interneurons of the SG that

inhibit the spino-thalamic tract. Serotonin is the main neurotransmitter of this path. Another important area of the descending pathway is the *locus coeruleus* by which originate noradrenergic fibres innervating the dorsal horn. Processes mediated by monoaminergic neurotransmitters such as norepinephrine, serotonin and dopamine modulate pain signalling within the dorsal horn, although some of these neurotransmitters can exert either antinociceptive or pronociceptive effects, depending upon the subtype and location of the receptors involved (Benarroch 2008).

Descending serotonergic pathways can inhibit nociceptive signalling via 5-HT_1 receptor activation. Specifically, activation of 5-HT_{1A} receptors inhibits the excitability of spinothalamic projecting neurons and excitatory (i.e., pain facilitatory) interneurons (Bannister et al. 2005). Similarly, 5-$HT_{1B/D}$ receptor activation produce antinociception through inhibition of neurotransmitter release from primary nociceptive afferents (Benarroch 2008). In contrast, descending serotonergic pathway activation can promote nociceptive transmission by activating 5-$HT_{2/3}$ receptors.

Dopaminergic pathways can either inhibit or facilitate nociceptive signalling. Descending dopaminergic pathways inhibit nociceptive signalling by activating D_2 and D_3 receptors on primary nociceptive afferents and neurons in the dorsal horn, thus inhibiting pre-synaptic neurotransmitter release (Fleetwood-Walker et al. 1988; Benarroch 2008). However, dopamine can be pro-nociceptive if it activates D_1 spino-thalamic projecting (i.e., ascending) neurons (Coffeen et al. 2008). In contrast to serotonergic and dopaminergic receptor-mediated activity, each of which have pro- and antinociceptive effects, descending noradrenergic pathway

activation is only known to have antinociceptive effects (Benarroch 2008). Descending noradrenergic pathways, projecting to the spinal dorsal horn, originate from several areas within the pontine region of the brain (Hentall et al. 2003), and inhibit pain signalling by activating α_{2A} receptors on terminals of primary nociceptors, or by activating post-synaptic α_1 receptors, causing release of inhibitory neurotransmitters GABA or glycine from inhibitory interneurons (Benarroch 2008).

Pain Management

Different pharmacological interventions through distinct analgesic mechanisms of action

Pain relief can be reached through the use of various analgesic drugs, each having distinct mechanisms and sites of antinociceptive activity. In fact, the multitude and complexity of neuronal mechanisms that contribute to the pain transmission and modulation provide several possible targets (Fig. 1.2) for pharmacological intervention (Argoff 2011).

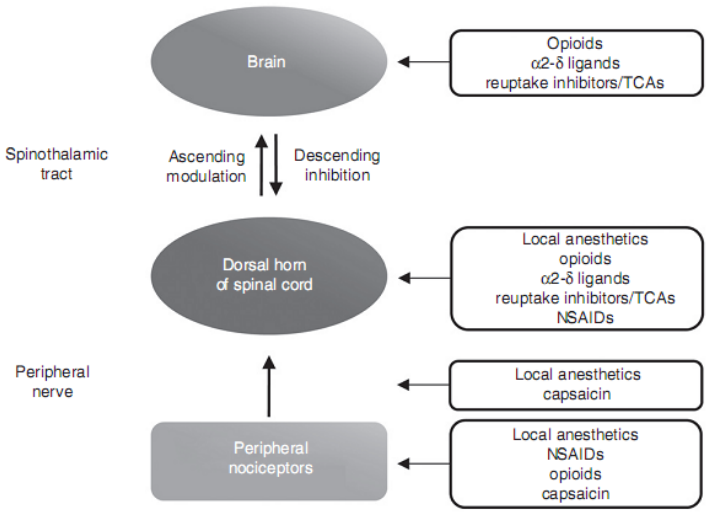
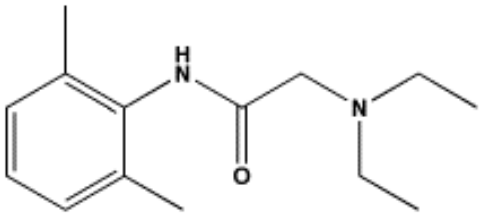
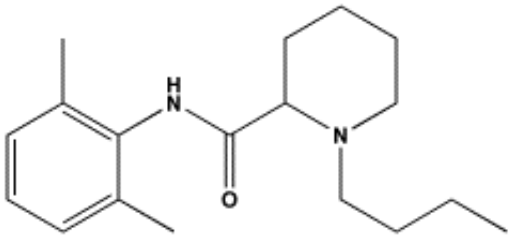


Figure 1.2. Mechanisms involved in the endogenous painful stimuli inhibition and related possible pharmacological intervention.

Moreover, for specific kinds of pain states some classes of drugs may be more effective than others. Therefore, it is important a full understanding of the mechanisms by which each drug class produces analgesia in order to make an appropriate analgesic drug's selection. For instance, *lidocaine* and *bupivacaine* are recommended for the neuropathic pain and the post-herpetic neuralgia (PHN) management. *Lidocaine* and *bupivacaine*, known as local anaesthetics, inhibit nociceptive signal conduction by blocking of neuronal membrane sodium channels at the site of application.



Lidocaine



Bupivacaine

Local anaesthetics are also effective in the management of acute post-surgical pain (Fig. 1.3), although the most commonly used drugs for the treatment of acute and chronic musculoskeletal and post-surgical pain are the NSAIDs. The NSAIDs' mechanism of action consists in the inhibition of the COX enzymes with a consequent reduction of inflammatory mediators such as the prostaglandins. For certain NSAIDs a further mechanism seems to be involved in the analgesic effect and consist in the block or inhibition of acid-sensitive ion channels within membranes of nociceptive neurons.

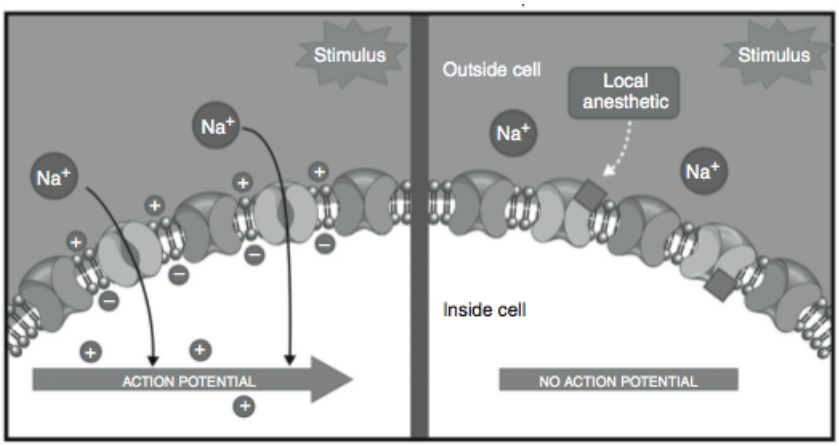
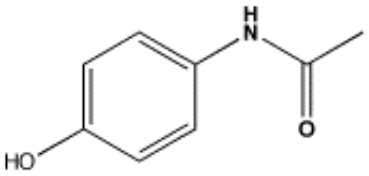
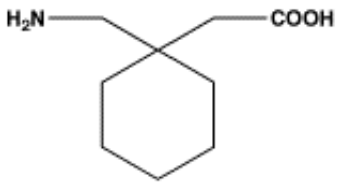


Figure 1.3. Local anaesthetics exert their antinociceptive effect by blocKing sodium (Na^+) channels within neuronal membranes, thus inhibiting afferent neuronal excitability and the propagation of nociceptive input via action potentials

NSAIDs as monotherapy showed suitable analgesia for mild to moderate pain, but in cases of severe pain they are used as adjunct remedy. *Acetaminophen* share with NSAIDs the analgesic and antipyretic properties but lack of anti-inflammatory activity due to its ability to inhibit the COX_3 enzyme distributed to the SNC. In addition, *acetaminophen* has been suggested to act on the serotonergic inhibitory descending pathway and the endogenous opioid pathway.



Acetominophen



Gabapentin

For severe pain management *acetaminophen* is used in multidrug therapies. For example, *acetaminophen* in combination with various *NSAIDs* produces a satisfactory pain relief for the treatment of post surgical pain with a side effects profile comparable to those of each single constituent in a monotherapy regimen. Some anticonvulsant drugs are

also effective analgesics and are often used in the management of neuropathic pain, diabetic neuropathy and PHN. The so-called *α2-δ ligands* bind to the α2-δsubunit of voltage dependent calcium channels within neuronal membranes inhibiting the release of excitatory neurotransmitter by pre-synaptic neurons (Fig. 1.4).

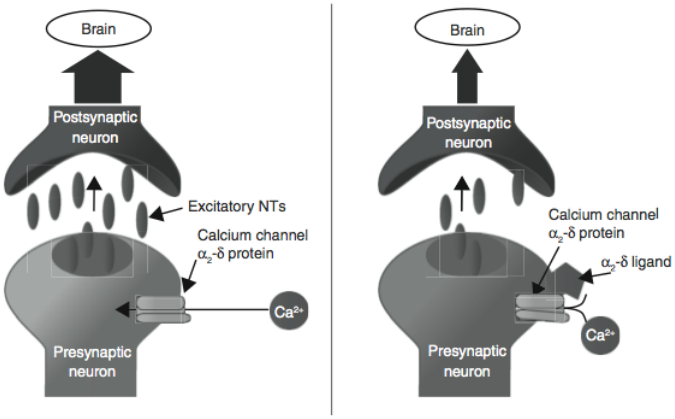


Figure 1.4. The α2-δ ligands are believed to promote analgesia by binding to the α2-δ subunit of voltage-dependent calcium channels on presynaptic neurons and inhibiting the release of excitatory neurotransmitters.

In addition, α2-δ ligands increase the spinal norepinephrine concentration by the activation of the descending inhibitory pathways.

In patients with chronic neuropathic pain, α2-δ ligands induce a 50% reduction of the pain intensity and the principle side effects are dizziness, somnolence, peripheral oedema, headache and dry mouth. In this context, a new compound to enter the pain market is *Gralise* (extended release gabapentin; Depomed), a once-daily formulation of the anticonvulsant *gabapentin*. In one recent study, gabapentin prevented acute pain after a Caesarean section (Moore et al 2011).

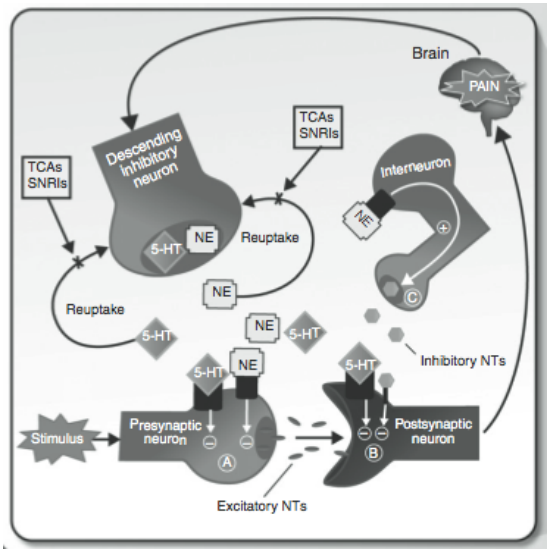
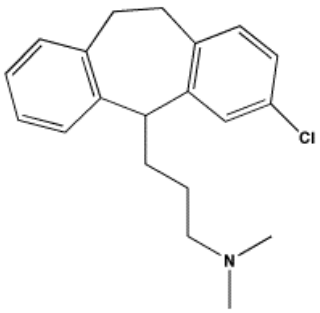


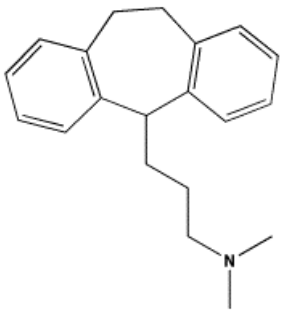
Figure 1.5. Monoamine re-uptake inhibitors such as tricyclic antidepressants (TCAs) and serotonin and norepinephrine re-uptake inhibitors (SNRIs).

Neuropathic pain is also managed with monoamine re-uptake inhibitors that exert their analgesic effects increasing the activity of descending pain-suppressing pathways (Fig. 1.5).

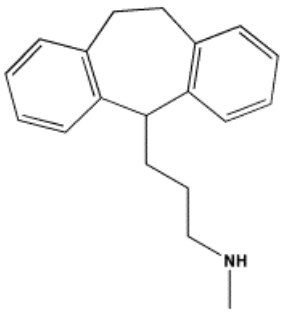
TCAs (e.g., amitriptyline, clomipramine, desipramine, imipramine and maprotiline), *SNRIs* (e.g., duloxetine and milnacipran) are also analgesic drugs belonging to the class of monoamine re-uptake inhibitors.



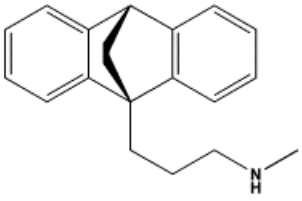
Clomipramine



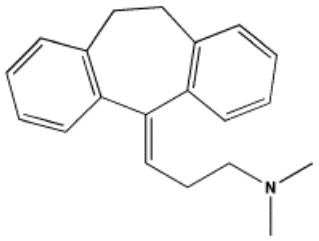
Imipramine



Desipramine

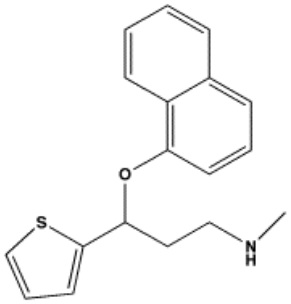


Maprotiline

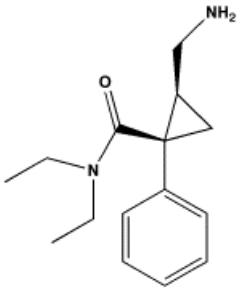


Amitriptyline

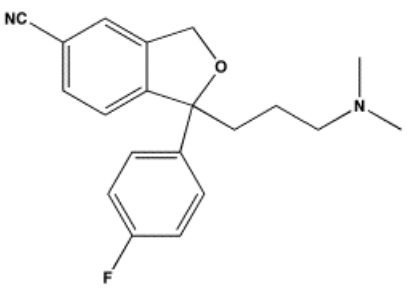
Studies performed in patients with neuropathic pain the use of *SSRIs* (Hempenstall and Rice, 2002) (e.g., citalopram, fluoxetine and paroxetine) showed no benefit, contrarily to TCAs and SNRI that provided significant pain relief in chronic pain states. However, their efficacy monotherapy for nociceptive pain is less well established and limited by anticholinergic side effects, including dry mouth, constipation, blurred vision, confusion, fatigue and potential cardiac toxicity.



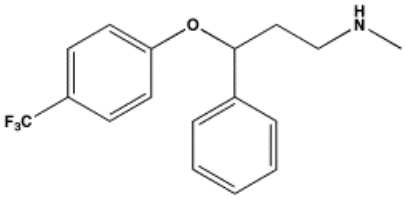
Duloxetine



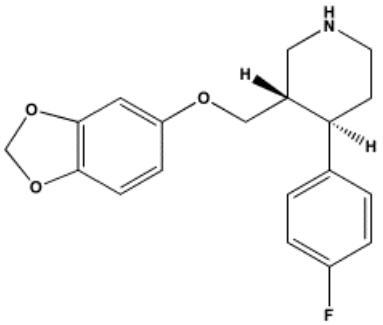
Milnacipram



Citalopram



Fluoxetine



Paroxetine

Opioids continue to be the mainstay of therapy against nociceptive pain which is pain caused by activation of peripheral afferent terminals by

noxious thermal, chemical or mechanical stimuli. Opioids work at various sites of the pain pathway inherent to the modulation of pain (Ballantyne and Shin, 2008). The analgesic effects of opioids are prevalently mediated through the activation of MOR in the CNS that can inhibit afferent nociceptive impulse transmission as well as activate the descending inhibitory pathways or suppress descending facilitatory pain pathways (Fig. 1.6). A part of the opioid-mediated analgesia – especially in painful inflammatory conditions - can be recognised to their ability to bind peripheral MOR also responsible of nausea, vomiting, constipation and pruritus. The analgesic properties of opioids are due to simulating endogenous properties of pain perception. Endogenous opioids - such as endorphins, encephalin, and dynorphins - interact with opioid receptors located in the hypothalamus (involved in temperature regulation and hormonal secretion), in the forebrain (involved in behavioural patterns including anxiety and expression of emotions).

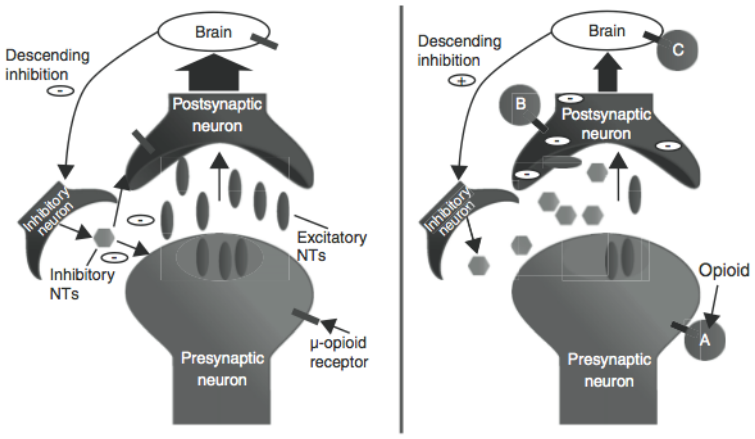
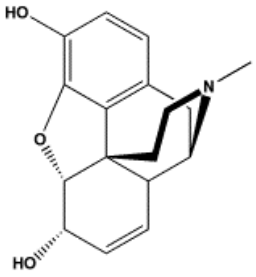
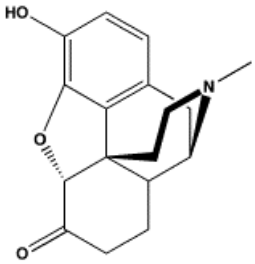


Figure 1.6. Opioids promote analgesia in a variety of ways, including: (A) decreasing pre-synaptic release of excitatory neurotransmitters, (B) decreasing post-synaptic neuronal excitability, and (C) promoting descending inhibition.

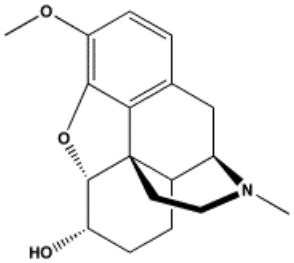
Opioids exhibit their analgesic effects by inhibition of calcium influx in the presynaptic membrane and substance P release and increasing potassium outflow. All these events determine hyperpolarization of presynaptic cells preventing nociceptive afferent information from spreading to adjacent neurons. *Morphine* is the prototype opioid agonist, which increases the threshold of pain perception. It binds strongly to the MOR, but also has agonistic effects at the KOR and DOR (Golembiewski and Rakic, 2010). Common effects of morphine are sedation, nausea, a feeling of body warmth, pruritus, urinary retention, euphoria, and decreased ability to concentrate. Constipation is the only side effect lacking tolerance (Carinci and Mao, 2010). Morphine can be administered i.m., i.v., s.c., rectally, epidurally, i.t., or orally.



Morphine



Hydromorphone



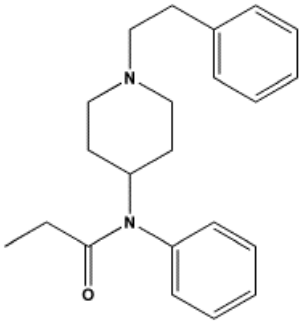
Codeine

Hydromorphone hydrochloride, a hydrogenated ketone of morphine, is a pure opioid agonist and its effects are similar to morphine. Hydromorphone may also cause dose-related respiratory depression, mood changes, mental clouding, euphoria, dysphoria, nausea, vomiting, and electroencephalographic changes. Like other opioids, hydromorphone long-term administration leads to constipation (Manchikanti et al. 2010).

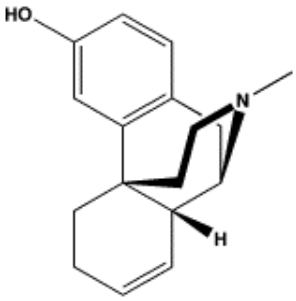
Codeine, a natural isomer of methylated morphine, is used for its

analgesic, antitussive, and antidiarrheal properties and represents the prototype of the weak to mid-range opioid.

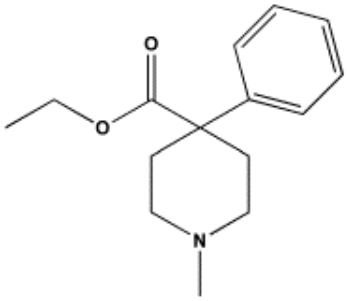
Fentanyl, a strong MOR agonist highly lipophilic, is administered both by transdermal and buccal routes. For chronic pain management the Fentanyl transdermal patch and the rapid-acting formulations, such as transmucosal and buccal tablets, are used.



Fentanyl



Levorphanol

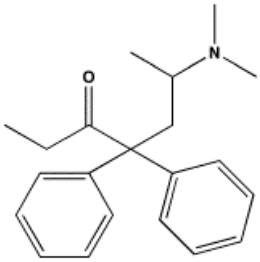


Meperidine

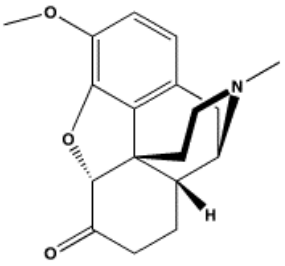
A potent synthetic opioid analgesic similar to morphine is *Levorphanol tartrate* that in addition to its primary effect at the opioid receptor has inhibitory effects both on NMDA receptors and serotonin and noradrenaline reuptake. Its NMDA actions seem to be valuable in neuropathic pain conditions where other analgesics fail.

Meperidine, known as *pethidine* is a rapid-acting synthetic opioid analgesic drug with potent anti-spasmodic proprieties due to its structural similar to atropine. Meperidine acts primarily as a KOR agonist and, in addition to anticholinergic effects, has local anesthetic activity related to its interaction with sodium ion channels.

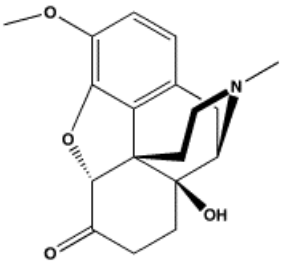
Meperidine is indicated for the treatment of moderate to severe pain, and is available as a hydrochloride salt in tablets, as syrup, or as im or iv injection.



Methadone



Hydrocodone



Oxycodone

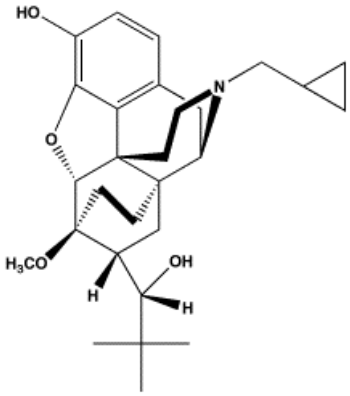
MOR interaction of *Methadone* produces morphine-like effects, and in addition it interact with voltage-gated potassium channels in the myocardium, which may lead to QT prolongation. Methadone is indicated for relief of severe pain and detoxification treatment. Moreover, preoperative treatment with a single dose of methadone, that has a long duration, improves post-operative pain in patients underwent complex spine surgery. Unlike morphine, methadone is a racemic mixture, one stereoisomer acts as a NMDA receptor antagonist that play an important role in the prevention of opioid tolerance, the other isomer is a MOR agonist.

Hydrocodone is structurally similar to codeine and produces effects most similar to morphine. It acts primarily at the MOR, but it is also a weak DOR and KOR agonist, usually combined with a weaker analgesic such as acetaminophen, ibuprofen, or aspirin.

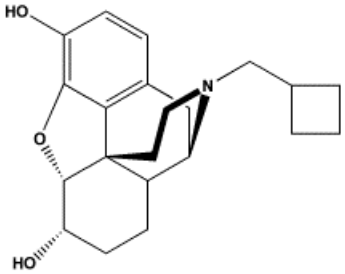
Oxycodone, with agonist activity *versus* MOR, DOR and KOR, is a potent synthetic opioid structurally similar to codeine and hydrocodone. Oxycodone is used as a single agent or co-formulated with paracetamol, ibuprofen, and aspirin.

Oxymorphone provides excellent analgesia with a lower incidence of sedation and higher patient satisfaction.

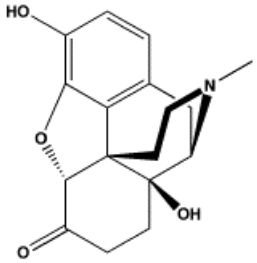
Buprenorphine is a thebaine derivative initially approved as an alternative to methadone for treatment of opioid addiction and moderate to severe chronic pain management. The mechanisms of action are incompletely understood but it results a partial MOR agonist and a weak KOR and DOR antagonist.



Buprenorphine



Nalbuphine



Oxymorphone

Nalbuphine hydrochloride is primarily a KOR agonist/MOR partial antagonist analgesic, also able to bind DOR.

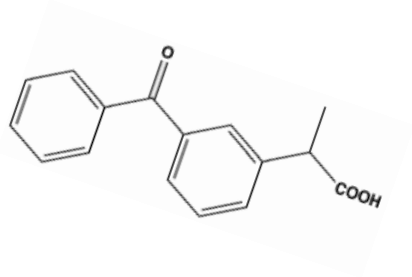
Opioid drugs' class have significant differences in pharmacokinetics, pharmacodynamics, pharmacology, mechanisms of activity, and disease specific utility. However, opioids are considered second-line therapy for chronic pain conditions, such as neuropathic pain, diabetic peripheral neuropathy and PHN (PrzewlocKi and Przewlocka, 2001). Evidence for their utility in these conditions is inconsistent and controversial (Pergolizzi et al. 2008). In fact, chronic pain is persistent and could not resolve over time, thus the long-term use of opioid analgesics for management of this pain conditions can lead to the exacerbation of detrimental side effects (Bekhit 2010), including tolerance to analgesic effect (Benyamin et al. 2008). Tolerance implies the dose escalation dose of the drug to achieve

and maintain constant analgesic effect (Ueda and Ueda, 2009; Grecksch et al. 2006).

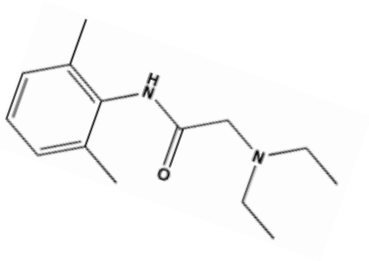
Multidrug analgesic approaches multiple mechanisms of pain signaling and modulation.

For the complexity of pain and the variety of physiological mechanisms involved a single analgesic agent may not provide optimal analgesia for certain types of pain. Thus, to enhance analgesia and/or reduce side effects the multidrug analgesic approaches or polypharmacology, otherwise the practice of combining different analgesic agents has been integrated into clinical practice for the treatment of both acute and chronic pain. As demonstrated in some clinical studies the multidrug therapy allow to reduce consummation of opioid analgesics and lowered the incidence of opioid-associated side effects (Christo and Mazloomdoost, 2008). Combining two or more analgesic drugs can produce either additive or synergistic effects. The analgesic effect resulting from the multidrug administration is said additive if the analgesia produced by the drugs combination is similar to that expected, evaluated considering the dose-response curves of each drug. Differently the analgesic effect resulting from a regimen of multidrug administration is said synergistic if the effect of the combination is greater than expected. The total dose of the synergistic combination can be lower than that of single analgesic agents, diminishing the risk of adverse effects associated with each drug. Different clinical trials performed on patients that underwent minor surgery revealed for the combination of NSAID with systemic opioids additive or synergistic analgesic effects, demonstrating the ability of additional NSAID

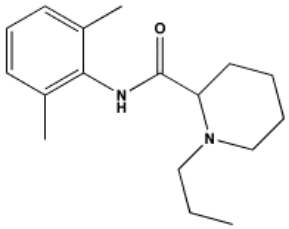
to reduce the dose of opioid with subsequent reduction of opioid-induced side effects. The co-administration of an NSAID, like indomethacin or ketorolac, to an opioid resulted to a better analgesia for the post-operative pain relief. As well as the combination of systemic NSAID with intrarticular bupivacaine reduced pain and analgesic requirements.



Ketoprofen

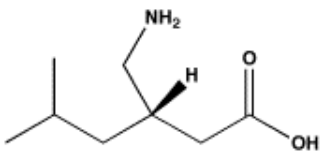


Lidocaine

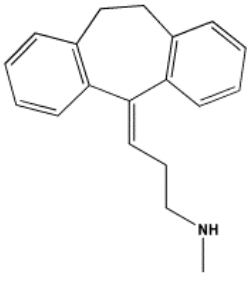


Ropivacaine

Similarly, the combination of ketoprofen with lidocaine reduced opioid analgesic requirements. Moreover, the intra-articular co-administration of ropivacaine, morphine and ketorolac lowered post-operative pain and morphine consumption. All these clinical trials seem to establish that peripheral-acting analgesics may be an important component of multimodal pain therapy. An improved analgesic efficacy in patients with moderate to severe neuropathic pain was reported for a multidrug regimen consisting of oxycodone and pregabalin. Experimentally, it was demonstrated that local anesthetics intrathecally administered potentiate spinal morphine antinociception.



Pregabalin



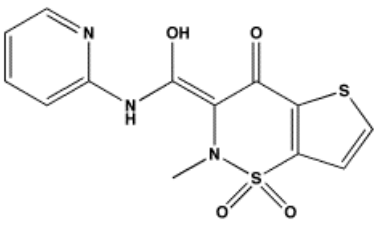
Nortryptiline

The additive or synergistic analgesic effects produced by the co-administration of spinal bupivacaine and morphine is the consequence of conformational changes in spinal opioid receptors that lead to a decreased binding to MOR but increased binding to DOR and KOR.

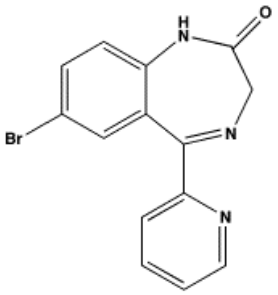
Combination of α2-δ ligands, such as gabapentin or pregabalin, with nortriptyline or opioid analgesic, like morphine or oxycodone, provided better pain relief than the corresponding single-drug therapy in patients with PHN.

Moreover, recent experimental studies demonstrated additive effects between intrathecal morphine combined with α-adrenergic agonists such as norepinephrine, carbacol, or midazolam. In fact, in several chronic pain states the combination of monoamine-reuptake inhibitors with opioid analgesics seems to play a benefit role.

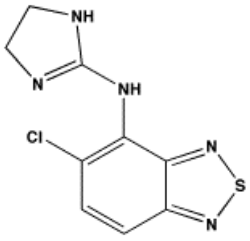
Other examples of effective combination drug therapies are the association of tramadol and acetaminophen, or tenoxicam and bromazepan, or fluoxetine and amitriptyline for pain associated with fibromyalgia, tizanidine and amitriptyline for chronic tension-type headache, and gabapentin and amitriptyline for chronic pelvic pain.



Tenoxicam



Bromazepan



Tizanidine

A valid analgesic effect results by the co-administration of two opioid drugs such as oxycodone and naloxone (Hermanns et al. 2012). In fact, a number of experimental data and clinical trials proved that the association of a MOR agonist/DOR-agonist – as well as a MOR-agonist/DOR-antagonist – improved the analgesia MOR-induced reducing the incidence of side effects by the reducing the requirement of MOR activation. In the clinical practice, the oxycodone/naloxone association reduces the development of the constipation maintaining a suitable analgesic effect.

Numerous guidelines for pain management supported the use of combination therapy in clinical practice. As demonstrated by a number of clinical studies, multidrug analgesia offer beneficial effects compared with single agent regimens.

To avoid the potential complications associated with polypharmacology, a single analgesic agent that act on multiple pain pathways through different mechanisms could represent a valid possibility for pain management.

For instance, the current standard treatment for neuropathic pain (Fig. 1.7) condition is the anticonvulsant *pregabalin* (Lyrica; Pfizer) that acts as agonist either of the GABA receptors and α 2δ subunit, and alters neuronal activity through modulation of the calcium channels. Despite this dual mechanism of action is valid in many neuropathic pain conditions, it is associated with several CNS-related side effects such as sedation and dizziness. For the treatment of PHN the anti-depressant

duloxetine (Cymbalta; Eli Lilly) is a multitarget agent that combines the dual ability to inhibit the serotonin and noradrenaline reuptake.

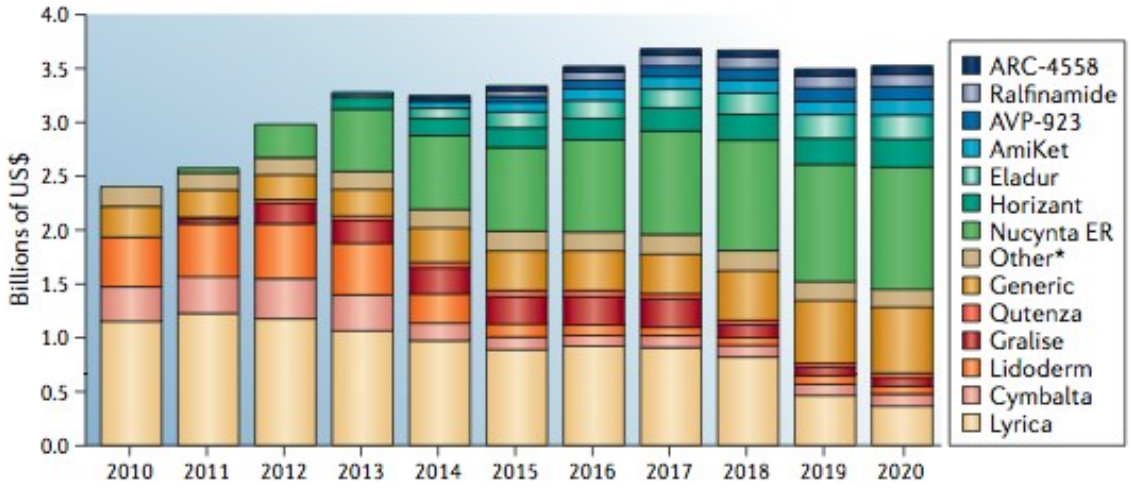


Figure 1.7. The neuropathic pain market. Data are for the seven major markets (the United States, Japan, France, Germany, Italy, Spain and the United Kingdom).

Another example of multitarget drug of newest enter in the pain market is *Nucynta ER* (extended release tapentadol; Johnson & Johnson) possessing a dual mode of action consisting of agonism of the MOR and inhibition of noradrenaline reuptake, and has demonstrated efficacy in both neuropathic and nociceptive pain conditions.

Nucynta is prescribed for conditions involving both acute and chronic pain, in fact its opioid-sparing effects and reduced potential for abuse could represent a clinical advantage in chronic pain conditions requiring long-term management. *

* **ABBREVIATIONS**: MOR, μ-opioid receptor; DOR, δ-opioid receptor; KOR, κ-opioid receptor; CNS, central nervous system; SG, gelatinous substance; PAG, periaqueductal grey; RMN, raphe magnus nucleus; GABA, γ-aminobutyric acid; PHN, post-herpetic neuralgia; NSAIDs, non-steroidal anti-inflammatories; COX, cyclooxygenase; TCAs, tricyclic antidepressants; SNRIs, serotonin and norepinephrine reuptake inhibitors; SSRIs, selective serotonin reuptake inhibitors; NMDA, *N*-methyl-*D*-aspartate; i.m., intramuscular; i.v., intravenous; s.c., subcutaneous; i.t., intrathecal.

Chapter 2

MULTITARGET LIGANDS

From multidrug combination therapy to the multitarget approach.

For the treatment of some pain conditions drugs having biological activity at a single target could be insufficient. Recently the medicinal chemistry research focused on ligands possessing multiple activities, known as multitarget drugs. Compared with the multidrug combinations, the multitarget ligands could show several advantages, such as the more predictable pharmacokinetic and pharmacodynamic, improved patient compliance and a lower risk of drug–drug interactions (Berger and Whistler, 2010; Burgess and Williams, 2010).

In the past the medicinal chemistry research was aimed on the paradigm "one-target, one-disease". However, nowadays is always more growing the idea to consider the design of agents that modulate multiple targets simultaneously, with the goal to enhance efficacy or improve safety respect to drugs addressed only a single target.

Several advantages, resulting from the clinical use of two or more drugs for the pain conditions management (Fig. 2.1 panel A), led to multicomponent drugs whereby two or more agents were co-formulated in a single tablet to make dosing regimen simpler and to improve patient compliance (Fig. 2.1 panel B) (Morphy and Rankovic, 2005).

For multicomponent drugs and multidrug combination approach there are significant clinical disadvantages. Indeed, main risks are related to differences in the relative rates of metabolism that could produce highly

complex pharmacokinetic/pharmacodynamic relationships leading to unpredictable variability.

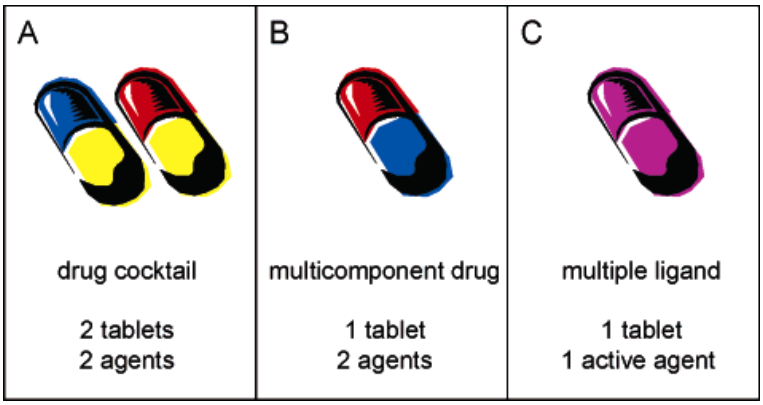


Figure 2.1. Clinical situations for multitarget therapy.

The multitarget ligand approach - consisting in the development a single chemical entity able to modulate multiple targets simultaneously (Fig. 2.1 panel C) - could represent a suitable alternative to the multicomponent drugs and multidrug combination approach. Risk of drug-drug interactions with multitarget ligands could be lower and their development, in terms of the risks and costs involved, is in principle no different from that of any other single entity.

Three medicinal chemistry strategies are possible to design multitarget drugs (Fig. 2.2).

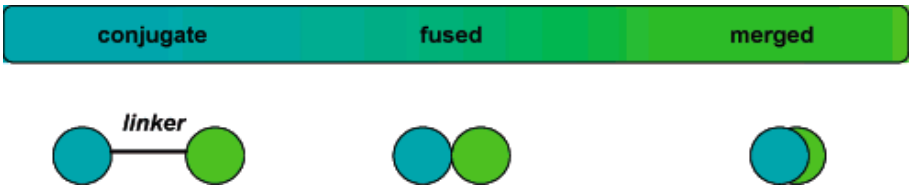


Figure 2.2. Three medicinal chemistry strategies to design multitarget ligands.

One of those consists in the connection of two or more pharmacophores directly (fused) or via a linker (conjugate) and the resulting ligand is appropriately named multivalent ligand. In this case, to design multivalent ligands should be considered the existing SAR of the components, with particular attention to the attachment sites of the pharmacophores to be connected.

On the basis of the design and synthesis of multivalent ligands, containing two or more distinct pharmacophores linked via a spacer, there is the expectation that they might be able to interact simultaneously with two receptor binding sites such as, for example, in a receptor heterodimer. However, when incorporated into the multivalent construct, the pharmacological characteristics (e.g. the receptor binding affinity and/or efficacy) of each pharmacophores may be altered. For instance, Portoghese et al. (1986) reported a range of bivalent opioid compounds with varying linker length designed with the aim to investigate pharmacodynamic and organizational features of opioid receptors.

Others medicinal chemistry strategies to design multitarget compounds are the so-called overlapping pharmacophores and the highly integrated pharmacophores approach (merged). Multitarget ligands based on highly integrated pharmacophores have been discovered by chance or from screening of compound libraries.

Multitarget analgesic ligands

As previously reported, the combination of two or more drugs producing their biological effects by interacting with two or more targets (multidrug

therapy) is a suitable and possible strategy widely used for the acute and chronic pain clinical management (Argoff 2011). From the numerous advantages providing by multidrug therapy, in the drug discovery process was established the "*one molecule-multiple targets*" approach. Besides an improved analgesic profile coupled to a less incidence of side effects, multitarget ligands possess favourable pharmacokinetic and pharmacodynamic proprieties.

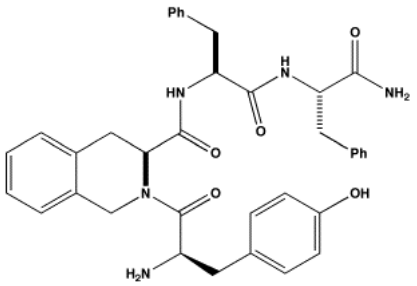
Pain sensation involves multiple signalling and modulatory pathways, employing a variety of neurotransmitters and other mediators (Argoff 2011). For instance, in the descending pain pathways endogenous opioids are key mediators. As well as, the NE, 5HT and DA - depending on receptor type and location - can positively or negatively modulate pain signalling. Thus, single analgesic therapies may be limited in their ability to comprehensively target these mechanisms. Considering that all mediators involved in pain signalling could represent potential targets for pharmacological interventions, multitarget ligands with a mechanism of action opioid-opioid and opioid-non opioid could be potential drug candidates for the management of different pain states.

This change from "*one molecule-one target*" to "*one molecule-multiple targets*" has several advantages compared to two drugs that are administered separately (Morphy and Rankovic, 2005). This trend in the drug discovery process has already been reflected in a number of analgesic ligands that modulate different protein targets at the same time.

Opioid-opioid multitarget ligands

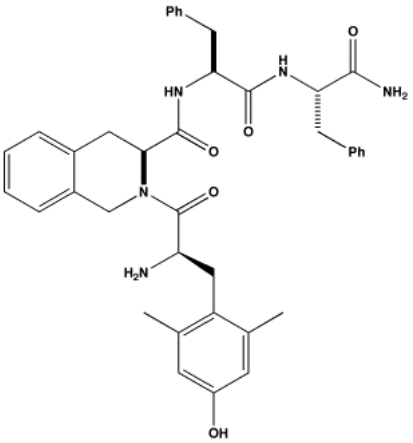
Most of clinical opioid analgesics, such as morphine, act via of MOR whose activation determine besides the analgesic effect also side effects - inhibition of gastrointestinal motility, respiratory depression, tolerance and physical dependence, often limiting their use in long-term treatment (Eguchi 2004; Bodnar 2010). Moreover, despite the potent analgesic profile, the use of KOR agonists is limited by their attitude to induce sedative, dysphoric and psychotomimetic effects. DOR agonists produce less physical dependence, respiratory depression and constipation than morphine but result weak analgesics and some of them produced convulsions in animals. Thus, selective agonists for MOR, DOR and KOR have limitations because of their side effects and/or weak analgesic activity.

Several evidences indicated that a multitarget opioid ligand could have therapeutic potential as potent analgesics with reduced side effects. TIPP-NH_2 (a) was the first compound having a MOR agonist/DOR antagonist profile. It displayed a moderate MOR affinity (K_i = 78.8 nM) and a high affinity for the DOR (K_i = 3.0 nM) (Schiller et al. 1992). In GPI assay, TIPP-NH_2 exhibited a moderate MOR agonist potency (IC_{50} = 1.70 μM) and a high DOR antagonist activity in MVD assay (K_e = 18.0 nM) (Schiller 1999). With the aim to improve the MOR affinity was synthesised the DIPP-NH_2 (b) peptide that possessed a greater MOR affinity and potency for both MOR and DOR, maintaining the efficacy profile of the parent compound.

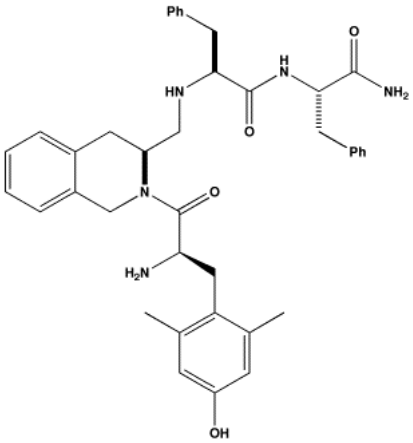


(a) TIPP-NH_2

DIPP-NH_2[Ψ] (c) displayed a better ratio of MOR and DOR affinities (K_i = 0.94 and 0.44 nM, respectively) and in the GPI assay was more potent as a MOR agonist (IC_{50}= 7.71 nM) but still retained a high DOR antagonist activity (K_e = 0.53 nM) in the MVD assay. In the rat tail-flick test, given i.c.v. DIPP-NH_2[Ψ] was more potent than morphine and produced a lower analgesic tolerance and no physical dependence in regimen of chronic administration.

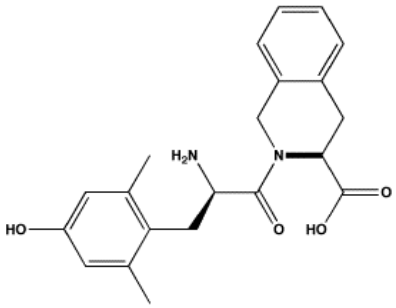


(b) DIPP-NH_2



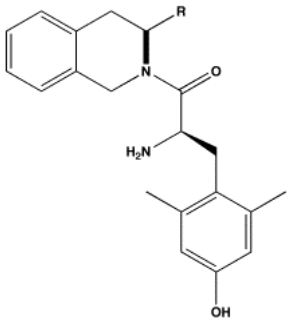
(c) DIPP-NH_2[Ψ]

Balboni et al. (2002) synthesised a series of multitarget Dmt-Tic analogs. All synthesized compounds (Fig. 2.3) showed a similar DOR binding affinity compared to the reference compound Dmt-Tic-OH (d) ($K_i \leq 0.13$ nM).



Dmt-Tic-OH (d)

Derivative e.2 exhibited a DOR/MOR agonist activity, while compounds e.3 and e.4 exhibited potent DOR antagonist and MOR agonist activities.



(e)

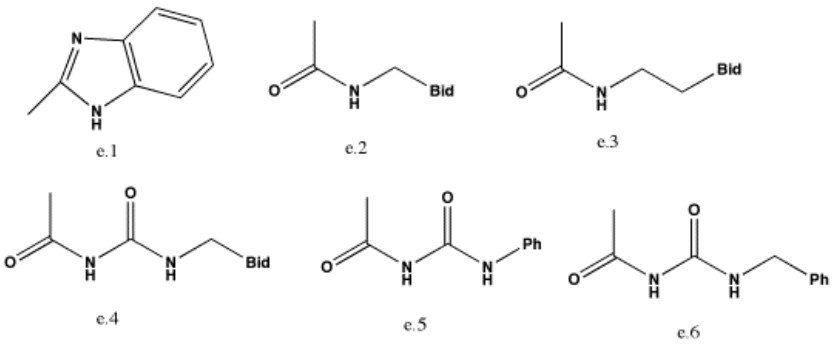


Figure 2.3. Dmt-Tic analogs structures.

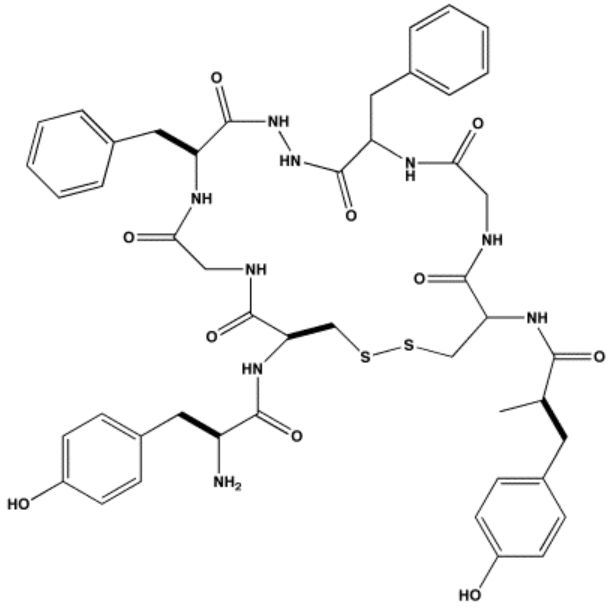
Endomorphin-1 and endomorphin-2 were investigated with the purpose to develop MOR/DOR ligands. The substitution of Tyr^1 with Dmt in these opioid peptides enhanced the binding affinities and functional potencies *versus* MOR and DOR (Salvadori et al.1995; Bryant et al. 1998; Sasaki et al. 1999). For instance, the ligand H-Dmt-Pro-Phe-NH-C_2H_4-Ph showed high MOR and DOR affinities (K_i = 0.51 and 18.0 nM, respectively) and

potent MOR agonist activity (GPI, IC_{50} = 5.03 nM) and a moderate DOR antagonist activity (MVD, pA_2 = 7.05).

The peptide H-Dmt-Tic-Gly-NH-Bzl named UFP-505, as tested by radioligand binding studies and functional assays, behaved as a potent MOR agonist and DOR antagonist (Balboni et al. 2002).

Endomorphin-1 and endomorphin-2 analogues containing either 3-(1-naphthyl)-D-alanine (D-1-Nal) or the 3-(2-naphthyl)-D-alanine (D-2-Nal) residue at position 4 were synthesised (Fichna et al. 2007). A significant MOR agonist activity (GPI, IC_{50}= 15.8 nM) and a moderate DOR antagonist activity (MVD, K_e = 62.4 nM) were described for the peptide H-Dmt-Pro-Trp-D-1-Nal-NH_2. The replacement of the Phe residue in position 3 of [Dmt^1]EM2 with the Tmp led to a MOR agonist/DOR antagonist profile (Li et al. 2007).

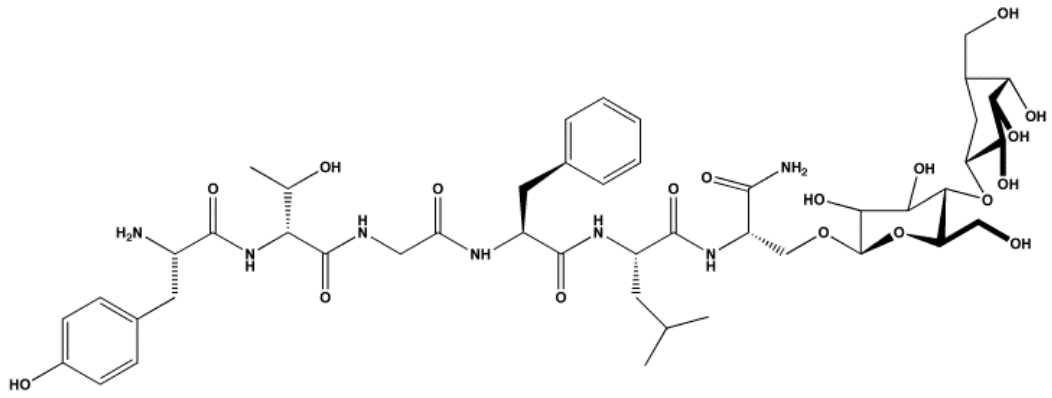
MOR/DOR ligands were also obtained through modification performed on encephalin peptide. For instance, the dimeric encephalin analogue Biphalin (Lipkowski et al. 1982), (H-Tyr-D-Ala-Gly-Phe-$NH)_2$) bind MOR and DOR receptors with high affinity (IC_{50}= 1.4 and 2.6 nM, respectively) and displayed MOR/DOR agonist activities (Horan et al. 1993). After systemic administration, Biphalin produced antinociceptive effect comparable to morphine, but induced no or lower dependence than morphine following chronic use (Lipkowski et al. 1996).



(f)

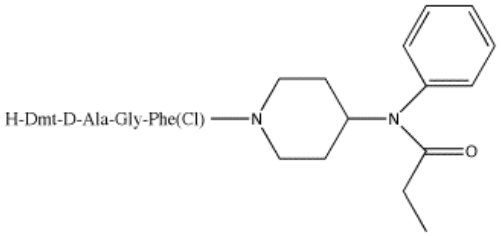
The piperazine biphalin derivative, H-Tyr-D-Ala-Gly-Phe-PPz<-Phe-Gly-D-Ala-Tyr-H, showed an excellent affinity *versus* MOR and DOR (IC_{50}= 0.48 and 0.65 nM, respectively) and possessed MOR/DOR agonist activities (GPI, IC_{50}= 2.5 nM; MVD, IC_{50}= 9.3 nM) (Mollica et al. 2005). Cyclic biphalin analogues were also synthesised and the most interesting peptide (f), exhibited better DOR/MOR affinities compared to biphalin (IC_{50}= 0.87 and 0.60 nM, respectively) maintaining its efficacy profile (Mollica et al. 2006).

MMP-2200, a glycosylated encephalin analogue (g), produced dose-related antinociception blocked by the MOR-selective antagonist β-FNA and the DOR-selective antagonist NTI (Do Carmo et al. 2008). Compared with morphine, MMP-2200, at the antinociceptive dose, possessed a low propensity to induce locomotor stimulation, tolerance, and physical dependence in mice.



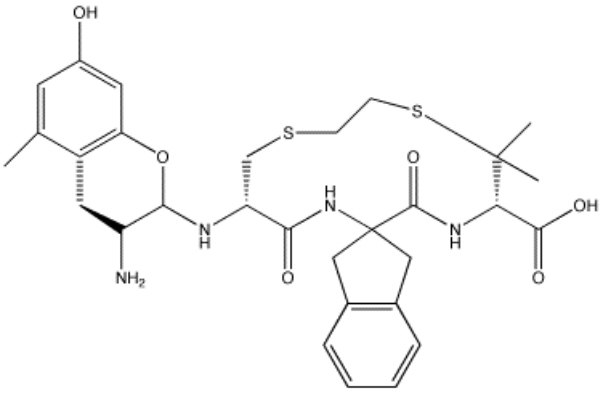
(g)

Moreover, encephalin-like tetrapeptides with a *N*-phenyl-*N*-piperidin-4-yl-propionamide moiety at the C-terminal resulted highly potent ligands of MOR and DOR (Lee et al. 2011). One of them (h) tested in an animal model of neuropathic pain, induced by the spinal nerve ligation, showed significant antiallodynic and antihyperalgesic effects.



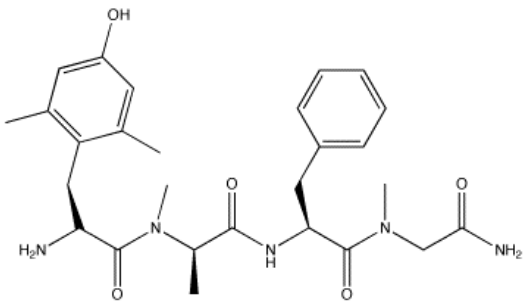
(h)

Purington et al. (2012) developed a series of potent non-selective opioid tetrapeptides having different efficacy at MOR and DOR. In particular, KSK-103 (i) bound with equal affinity to MOR and DOR (K_i=2.4 ± 0.7 nM and 2.3 ± 0.5 nM, respectively) and, as evaluated by [^{35}S]GTPγ S binding assay, it was a DOR antagonist and a MOR agonist with a greater potency than morphine (EC_{50}=4.7±0.7 nM, E_{max}= 59±11).

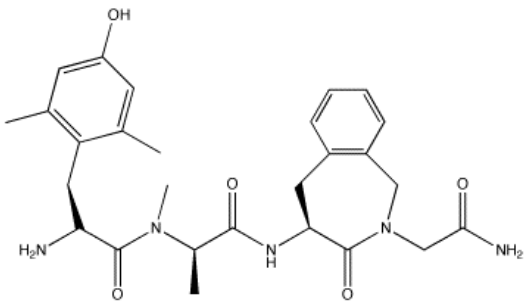


(i)

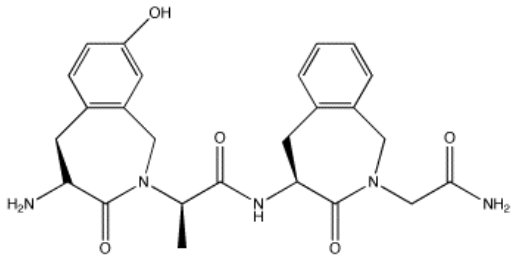
Recently, novel dermorphin tetrapeptides conformational constrained were also synthesised (Vandormael et al. 2011) (l, m, n, o). Most of these peptidic ligands displayed binding affinities in the nanomolar range for both MOR and DOR. Moreover, by measurements of cAMP accumulation and phosphorylation of extracellular signal regulated kinase were established their MOR/DOR agonist properties.



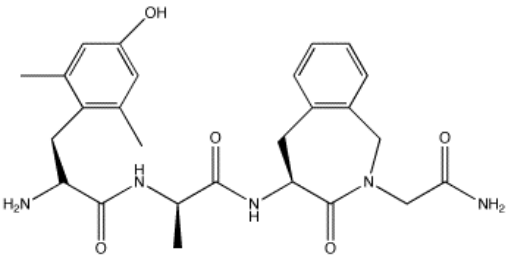
(l)



(m)



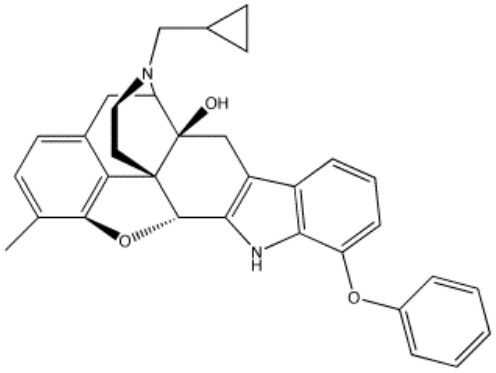
(n)



(o)

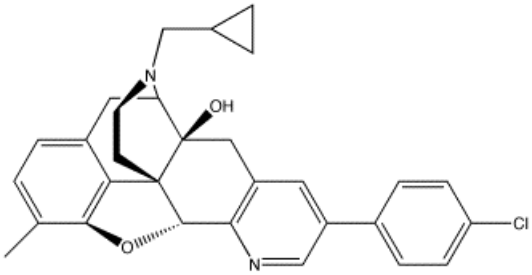
The first non-peptidic MOR agonist/DOR antagonist ligand was the NTI derivative, 7'-phenoxynaltridole (p), possessing a powerful DOR antagonist

activity (K_e = 0.25 nM in the MVD test) and a weak MOR agonist activity (IC_{50} = 450 nM in the GPI test) (Ananthan et al. 1998).



(p)

SAR studies on derivatives of NTX with a significant DOR selectivity (Takemori and Portoghese, 1992) allowed obtaining a series of pyrido- and pyrimidomorphinans (Ananthan et al. 1999). By this series SoRI 9409 (q), with mixed MOR and KOR agonist activities and DOR antagonist activity, showed a limited (or non-existent) antinociceptive tolerance induction capability.



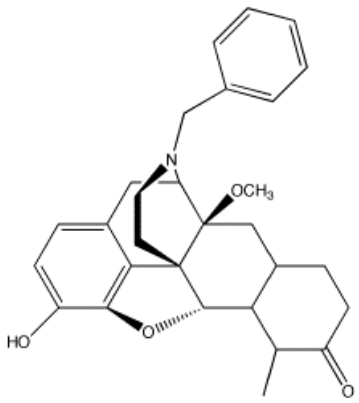
(q)

Compounds SoRI 20411 and SoRI 20648 were synthesised with the aim to increase the antinociceptive efficacy of SoRI 9409. Those compounds showed MOR agonist/DOR antagonist activities in the MVD and GPI assays confirmed also in the [^{35}S]GTPγ S binding assay (Ananthan et al.

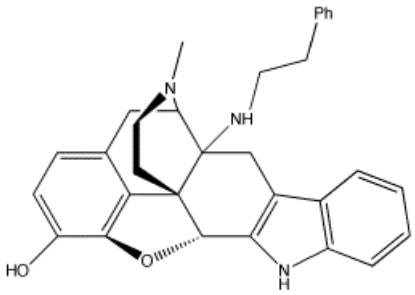
2004). SoRI 20648 was less effective in *In vivo* tests, while SoRI 20411 displayed a full MOR agonist activity.

By a series of 14-alkoxymorphinan-6-one derivatives emerged 14-ethoxymetopon ligand with a N-phenylethyl group (r) having higher MOR/DOR affinity and, in the hot-plate test, exhibited a greater antinociceptive potency than morphine (Lattanzi et al. 2005).

Modifications of oxymorphinindole, a selective DOR partial agonist, were made to obtain multitarget opioid analgesics (Grundt et al. 2003). The phenethylamine derivative (s) exhibited similar MOR (K_i= 1.68 nM) and DOR affinities (K_i = 1.10 nM) and was a full MOR agonist and a DOR partial agonist in the[^{35}S]GTPγ S assay.



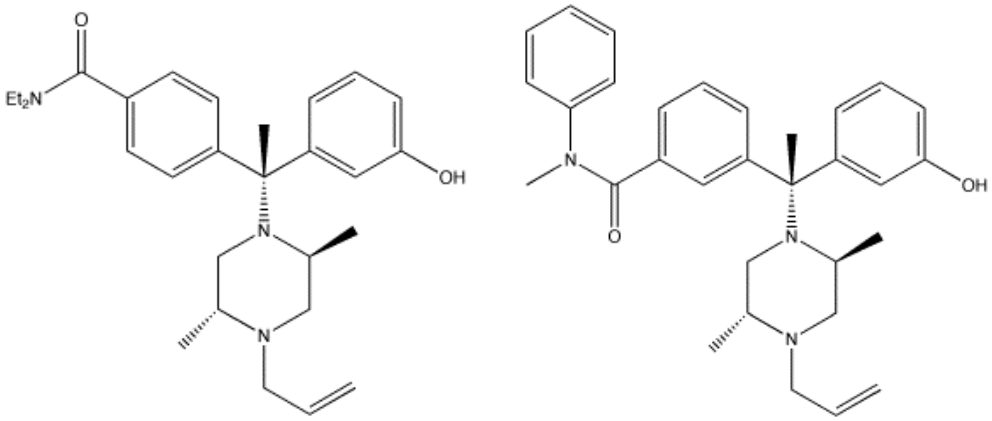
(r)



(s)

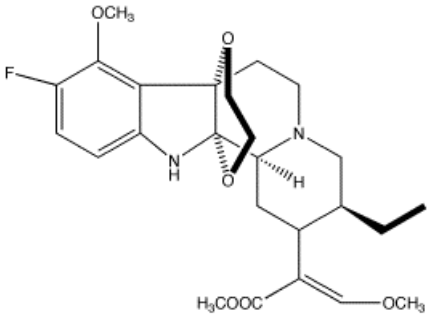
From compound BW373U86 (t), (Portoghese et al. 1988; Dondio et al. 1998) a potent and selective DOR agonist, a series of benzhydryl piperazine compounds were designed and synthesised (Bishop et al. 2003). Moving the diethylamide function of BW373U86 to the meta-position provided a MOR/DOR agonist with higher MOR activity than that of BW373U86 and decreased DOR activity. Replacement of the *m*-

diethylamide with *m*-*N*-methylanilide (u) induced an increase in the MOR/DOR activities (EC_{50}= 1.22 and 1.47 nM, respectively).



(t) (u)

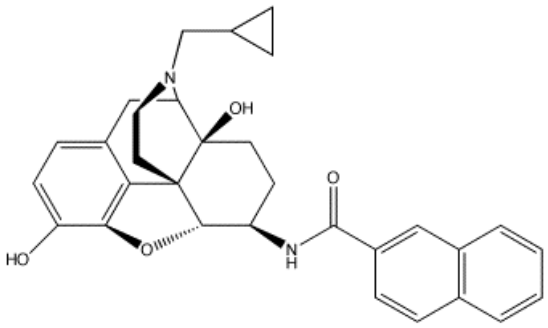
Derivatives of mitragynine, a major indole alkaloid isolated from the Thai medicinal plant *Mitragyna speciosa* possessing opium-like properties, were also synthesised (Matsumoto et al. 2008). In this context, MGM-9 (v) exhibited high affinity for MOR and KOR (K_i= 7.3 and 18 nM, respectively) and showed potent opioid agonistic proprieties *versus* MOR and KOR.



(v)

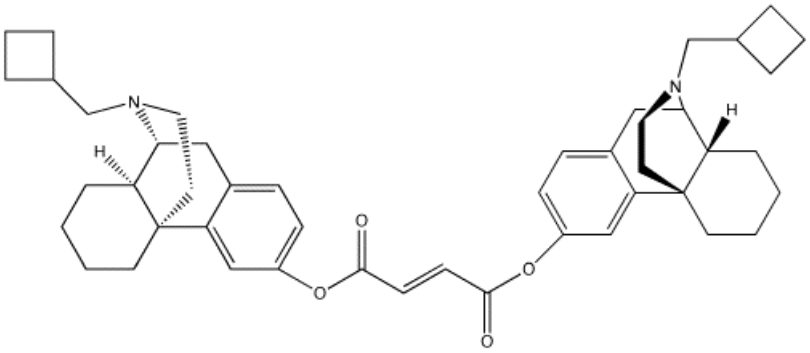
In mouse tail-flick s.c. and oral administration of MGM-9 produced potent antinociceptive effects and induced less hyperlocomotion and fewer rewarding effects than morphine. However, MGM-9 in regimen of repeated administration for five consecutive days induced antinociceptive tolerance.

N-naphthoyl-β -naltrexamine (z) selectively activated heteromeric MOR and KOR in HEK-293 cells and induced potent antinociception in mice without provoke tolerance (Yekkirala et al. 2011).



(z)

By a series of morphinan derivatives emerged the compound MCL-145 (a2) showing in radioligand binding studies nanomolar affinity *versus* KOR and MOR (K_i= 0.078 and 0.20 nM, respectively) (Mathews et al. 2005).

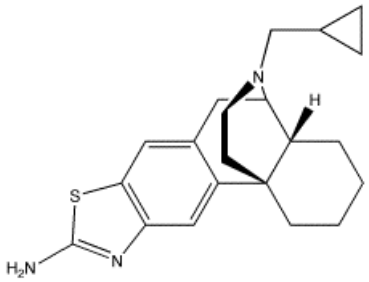


(a2)

In the [^{35}S]GTPγ S binding assay MCL-145 resulted a potent KOR/MOR agonist (EC_{50}= 4.3 nM E_{max}= 80% and EC_{50}= 3.1 nM E_{max}= 42%, respectively). Moreover, in the tail-flick test MCL-145 was equipotent to morphine.

ATPM (b2) compound acted as a full KOR agonist and a partial DOR agonist (Wang et al. 2009). Moreover, in comparison to (-)U50,488H, ATPM

demonstrated more potent antinociceptive effect and a less sedative effect. In relation to (-)U50,488H and morphine, ATPM in regimen of chronic administration showed less potential to develop antinociceptive tolerance.



(b2)

Opioid/non-opioid multitarget ligands

Based on the observation that an antagonistic effect on CCK_2 receptors blocks morphine tolerance, multitarget ligands possessing CCK_2 antagonistic and opioid agonistic activities were designed and synthesized. For instance, starting by the peptide named SNF-9007 (Fig. 2.4), that is a potent and highly selective CCK_2 agonist with a weak DOR agonist profile, were synthesised analogues in order to move the profile of efficacy *versus* CCK_2 antagonism (Slaninova et al. 1991).

Asp-*Tyr^1-D-Phe^2-Gly^3-Trp^4-NMeNle^5-Asp^6-Phe^7-NH$_2$*

Figure 2.4. Aminoacidic sequence of the SNF-9007 peptide. Pink aminoacids represent the opioid pharmacophore, the blue ones the CCK_2 pharmacophore.

[desAsp^0]-SNF-9007 (Neumeyer et al. 2000) analogue possessed an improved binding affinity at the DOR and MOR (K_i= 1 and 100 nM, respectively) and high binding affinity for CCK_2 receptors (K_i = 15 nM) and micromolar affinity at the CCK_1 receptors (K_i= 3,600 nM). In the functional assays, this compound displayed a potent agonist activity at the CCK_2 and

opioid receptors. Additional structural modifications led to the analogues Tyr^{1}-D-Phe^{2}-Gly^{3}-D-Trp^{4}-*N*MeNle^{5}-Asp^{6}-Phe^{7}-NH_2 and Tyr1-D-Ala^{2}-Gly^{3}-D-Trp^{4}-*N*MeNle^{5}-Asp^{6}-Phe^{7}-NH_2 displaying excellent affinities for the DOR and CCK_2 receptors; these compounds also had good MOR affinity.

MOR-CCK_2 ligands were synthesised using non-petidic nuclei that are oxymorphone for the MOR pharmacophore and L-365,260 for the CCK_2 antagonist pharmacophore linked by different spacer length (Fig. 2.5) (Zheng et al. 2009; Portoghese et al. 1986).

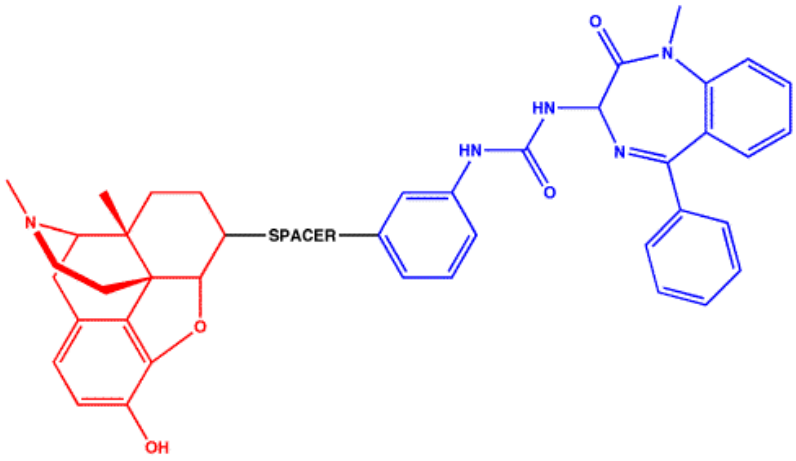


Figure 2.5. MOR-CCK_2 peptides. Red portion is the opioid pharmacophore (oxymorphone), the blue one is the CCK_2 antagonist pharmacophore (L-365,260).

Binding assays, performed on CHO cells expressing CCK_2 and MOR, showed that ligands having spacer lengths of 16 (d2) or 18 atoms (f2) (Fig. 2.6) possessed higher CCK_2-affinity in co-expressed cells (K_i= 57 and 33 nM) relative to cells that expressed only CCK_2 receptors (K_i= 356 and 71 nM).

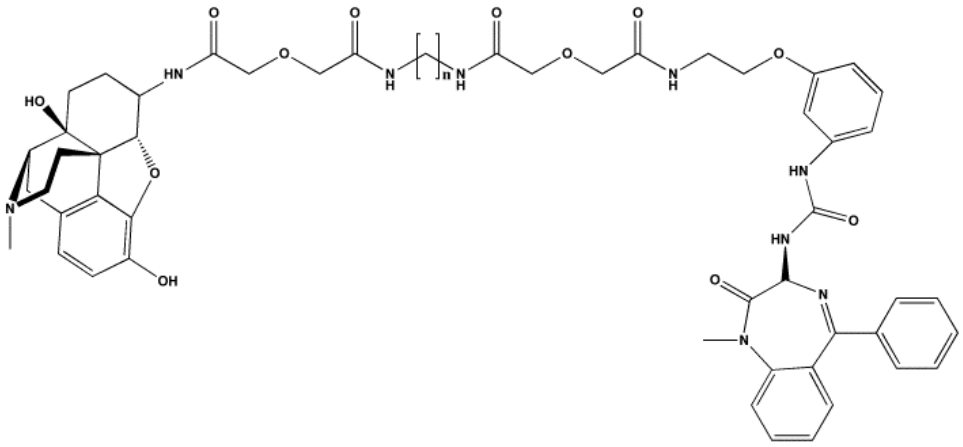


Figure 2.6. MOR-CCK_2 peptides. n=0 (d2) and n=2 (f2).

Both peptides revealed high affinities for the MOR in CHO cells expressing either MOR or CCK_2 receptors (K_i= 8.3 and 5.7 nM, respectively) and co-expressing MOR and CCK_2 receptors (K_i= 3.9 and 7.3 nM, respectively). *In vivo* experiments showed that those ligands did not produce tolerance.

Several evidences proved that a ligand able to simultaneously activate MOR and antagonise Substance P function by blocking its NK1 receptor, was a valid approach to produce a significant analgesic effect with a low incidence to tolerance induction (King et al. 2005; Misterek et al. 1994). The chimeric peptide AA501 (Boyle et al. 1994), with an opioid receptor agonist pharmacophore biphalin-related and the NK1 antagonist pharmacophore derivative of tryptophan connected through a hydrazide bridge (Fig. 2.7), revealed a reasonable MOR affinity (K_i= 80 nM) and a micromolar affinity for the NK1 receptors (K_i=5 μM).

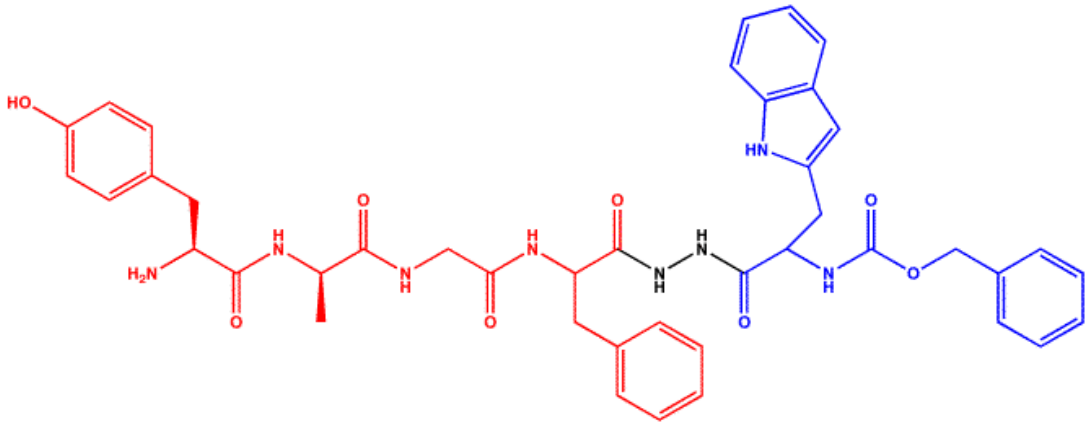
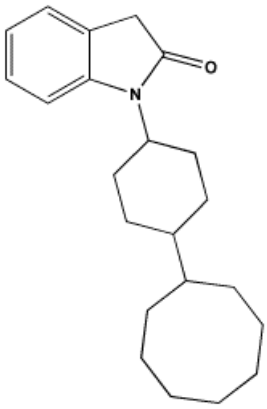


Figure 2.7. AA501 peptide. Red aminoacids represent the opioid pharmacophore, the blue ones the NK1 pharmacophore.

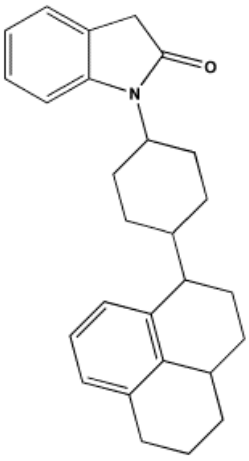
The compound H-Tyr-D-Ala-Gly-Phe-Phe^5-Pro-Leu-Trp-O-3,5-Bzl$(CF_3)_2$, known as TY003, exhibited a nanomolar affinity *versus* DOR and MOR (K_i = 15 and 28 nM, respectively) and a subnanomolar affinity for the NK1 receptors (K_i= 0.88 nM) (Yamamoto et al. 2007). The substitution of Phe^5 with Gly^5 in the TY003 peptide gave a compound with a lower DOR affinity and preserved MOR and NK1 receptors affinities. Other analogues of TY003 with Leu^5 and Met^5, besides to show better DOR affinity (K_i= 5.0 and 2.8 nM, respectively), retained a high affinity for the NK1 receptors (K_i= 0.8 and 0.29 nM, respectively). The oxidation of Met^5 led to an increased affinity for the NK1 receptors (K_i= 0.2 nM). The replacement of the Met^5 with the bioisostere Nle5 increased the DOR affinity (K_i= 1.8 nM) but the MOR and NK1 K_i values were increased. N-methylation at position 5 gave a compound that showed a significant decrease in binding affinity for the DOR (K_i= 77 nM) and MOR (K_i= 140 nM) while maintaining a high affinity for the NK1 receptors (K_i= 0.71 nM).

SR14150 (g2) and SR16835 (h2) are moderately selective nociceptin/orphanin FQ (NOP) receptor agonists (Khroyan et al. 2011). In

the [^{35}S]GTPγ S binding assay, SR14150 was a partial agonist at both NOP and MOR, whereas SR16835 was a full agonist at the NOP receptor with low efficacy *versus* MOR.



(g2)



(h2)

Using mice in chronic pain subsequent to spinal nerve ligation surgery, SR14150 and SR16835, administered s.c., had antiallodynic activity when mechanical allodynia was measured with von Frey monofilaments.

Analogously, Tramadol and its derivative Tapentadol (Wade and Spruill, 2009) combining weak MOR agonism and monoamine reuptake inhibition produced significant analgesic effect with low incidence of side effects MOR agonist induced. *

* MOR, μ-opioid receptor; DOR, δ-opioid receptor; KOR, κ-opioid receptor; SAR, structure-activity relationship; NE, norepinephrine; 5-HT, serotonin; DA, dopamine; GPI, guinea pig ileum; MVD, mouse vas deferens; i.c.v., intracerebroventricular; Tmp, 2',4',6'-trimethylphenylalanine; NTI, naltrindole; β-FNA, β-funaltrexamine; NTX, naltrexone; s.c., subcutaneous; NK, neurokinin; CCK, cholecystokinin; NOP, nocicetin/orphanin FQ peptide.

Chapter 3

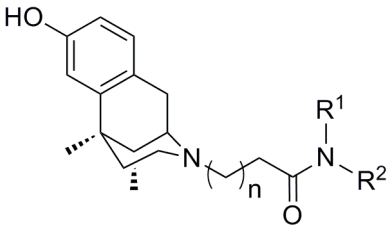
THE BENZOMORPHAN LIGAND LP1

Pharmacological evaluation of the benzomorphan-based compound LP1

Background

Despite MOR opioid analgesics are the standard treatment options for acute and chronic pain management, their long-term use induce the development of tolerance to the analgesic effect that could exacerbate other side effects (Brush 2012). The dissociation of analgesia from tolerance using MOR selective agonists is nearly impossible (Eguchi 2004; Bodnar 2010). Moreover, the design and synthesis of ligands highly selective for DOR and KOR as a strategy to overcome or limit MOR-mediated tolerance was unsuccessful. On the contrary, it has been demonstrated that ligands capable to target simultaneously different receptors could represent suitable candidates for the treatment of chronic pain (Dietis et al. 2009; Prezzavento et al. 2010; Schiller 2010). For instance, an improved antinociception and a low propensity to develop tolerance were reported for ligands possessing MOR-DOR agonist activity as well as MOR agonist-DOR antagonist activity.

Pasquinucci et al. (2010) described the synthesis and SAR of opioid ligands based on the 6,7-benzomorphan class. In particular, it was synthesised a new series of 5,9-dimethyl-2'-hydroxy-6,7-benzomorphan derivatives (12–22) by introducing different functional groups on the N-substituent such as an aromatic ring and/or alkyl residues linked by an N-propanamide or N-acetamide spacer (Fig. 3.1).



12-18 n = 1

R^1 = Ph, *c*-C_6H_{11}, Bn, CH_2-*c*-C_6H_{11}, *t*-Bu

R^2 = H, Me, Et

19-22 n = 0

R^1 = Ph, *c*-C_6H_{11}

R^2 = H, Me, Et

Figure 3.1. Structures of N-substituted 6,7-benzomorphan ligands 12–22.

Data obtained by competition binding assays confirmed the critical importance of the N-substituent that confers affinity and selectivity *versus* different opioid receptors subtypes. The most promising compound was 3-[(*2R,6R,11R*)-8-hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2H)-yl]-N-phenylpropanamide named LP1 (Fig. 3.2).

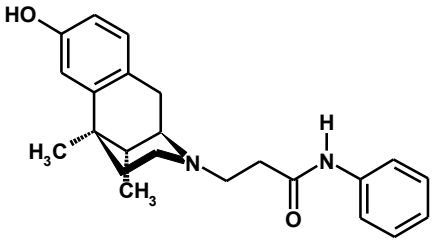


Figure 3.2. The benzomorphan-based compound LP1.

LP1 exhibited high and moderate affinity for MOR and DOR (Ki^{MOR}=0.83 ± 0.05 nM and Ki^{DOR}=29 ± 1 nM) (Table 3.1). In the tail flick test, the antinociceptive potency of LP1 was comparable to that of morphine

(ED_{50}=2.03 mg/kg s.c. *vs.* 2.7 mg/kg s.c.) and it was completely naloxone-reversed.

				Ki (nM) ± SEM[a,b]		
Comp.	n	R^1	R^2	MOR	DOR	KOR
12 (LP1)	2	H	Ph	0.83 ±	29.1 ± 1	110 ± 6
13	2	H	*c*-C_6H_{11}	56 ± 3	> 5,000	501 ± 25
14	2	Me	Ph	65 ± 3	> 5,000	261 ± 14
15	2	Et	Ph	136 ± 7	> 5,000	70 ± 4
16	2	H	Bn	105 ± 6	> 5,000	237 ± 13
17	2	H	CH_2-*c*-C_6H_{11}	107 ± 6	> 5,000	134 ± 7
18	2	H	*t*Bu	372 ± 18	> 5,000	422 ± 20
19	1	H	Ph	722 ± 40	> 5,000	> 5,000
20	1	H	*c*-C_6H_{11}	2,930 ±	> 5,000	612 ± 27
21	1	Me	Ph	1,370 ± 69	> 5,000	339 ± 17
22	1	Et	Ph	1,120 ± 66	> 5,000	335 ± 17
DAMGO				0.87 ± 0.6	2,670 ±	> 5,000

[a] Values are means ± SEM of three separate experiments, each carried out in duplicate.
[b] Ki values were obtained as [^{3}H]DAMGO displacement for the MOR, [^{3}H]DPDPE displacement for the DOR, and [^{3}H]U69,593 displacement for the KOR.

Table 3.1. Competition binding *versus* MOR, DOR and KOR of 5,9-dimethyl-2'-hydroxy-6,7-benzomorphan derivatives 12-22.

Aim

In light of the significant antinociceptive effect and interesting profile of affinities *versus* MOR and DOR of LP1, the purpose of this thesis was to study of the functional profile of LP1 through [^{35}S]GTPγS binding assay. To further assess the involvement of opioid receptors subtypes in LP1 effect, in the tail flick test were used selective antagonists for MOR, DOR and KOR, NLZ, NTI and norBNI, respectively (Bedini et al. 2010). To delineate the pharmacological profile of LP1, the effect of NX-M, a non-selective opioid receptor antagonist by i.c.v. or s.c. injection, was

investigated to determine whether the antinociception produced by LP1 was central or peripheral (Craft et al.1995). Moreover, it was assessed and compared the induction of tolerance to the antinociceptive effects of LP1 and morphine. Finally, LP1 was tested in a model of neuropathic pain - induced by CCI of the left sciatic nerve - and inflammatory pain - induced by i.pl. injection of carrageenan.

Experimental Procedure

Resolution of (-)-cis-(1R,5R,9R)-N-normetazocine

The starting material for the synthesis of the 6,7 benzomorphan-based compound LP1 was the (-)-*cis*-(*1R,5R,9R*)-*N*-normetazocine by the resolution of the optical isomer of the (±)-*cis*-N-normetazocine ((±)-(*2R/2S,6R/6S,11R/11S*)-6,11-dimethyl-1,2,3,4,5,6-exahydro-2,6-methan-3-benzazocin-8-olo) (Fig. 3.3).

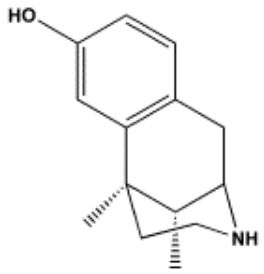
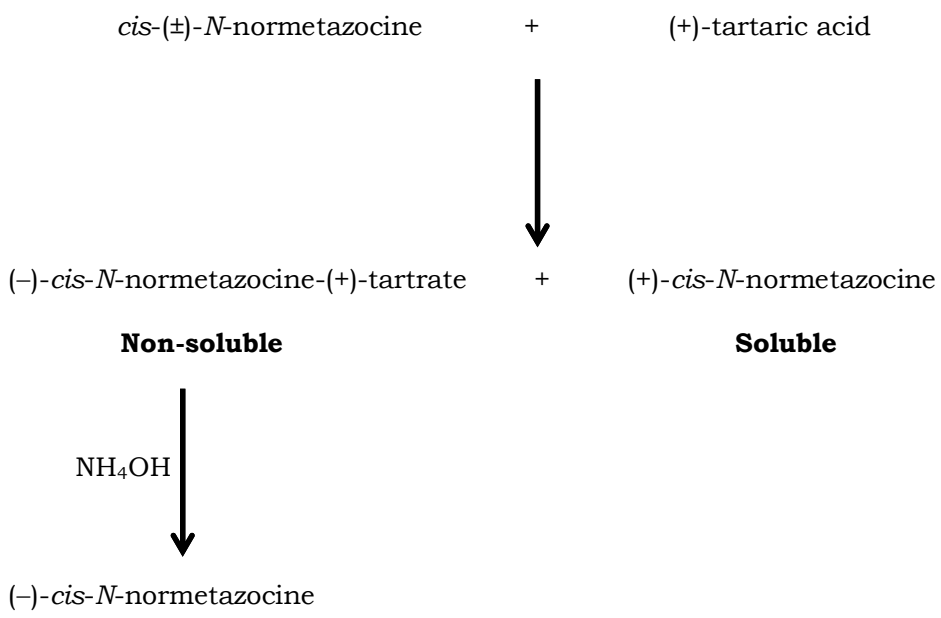


Figure 3.3.(-)-*cis*-(*1R,5R,9R)-N*-normetazocine structure.

Brine et al. (1990) reported and improved resolution procedure (Scheme 3.1) in which (±)-*cis*-*N*-normetazocine (90 mmol) dissolved in chloroform (138,6 ml) and ethanol (109,2 ml) was added under nitrogen to a solution of (+)-tartaric acid (22,5 mmol) in water (54,6 ml). The biphasic mixture resulting was refluxed for 21 hours, then was kept to 45°C for 6 hours and finally to rt overnight. By filtration was collected the crystallised (–)-

cis-*N*-normetazocine-(+)-tartrate that was washed with cold ethanol and dried under vacuum. It was obtained a white powder weighing 7.85 g with a melting point of 305°C. The (-)-*cis*-(*1R,5R,9R*)-*N*-normetazocine-(+)-tartrate salt was added to chloroform (70.68 ml), methanol (35.34 ml) and 20% aqueous ammonium hydroxide (53 ml). Aqueous layer was extracted three times with chloroform and the combined organic layers were washed with brine, dried over Na_2SO_4 anhydrous and concentrated on a steam bath and under a stream of nitrogen. In these conditions the base began to crystallise and the mixture was diluted with several portions of 2-propanol. The mixture was concentrated to a final volume of 80 ml and was kept to 4°C overnight.

Scheme 3.1. Resolution of (±)-*cis*-*N*-normetazocine



The resulting precipitate was collected by filtration and washed with several portions of 2-propanol and finally dried under vacuum obtaining

(–)-*cis*-*N*-normetazocine as a white powder weighing 3.74 g with a melting point of 260-261.5°C and [α]= -73° (Table 3.2). The (-)-*cis*-(*1R, 5R,9R*)-*N*-normetazocine was also characterised by IR and [^{1}H]-NMR (Fig. 3.4 and Fig. 3.5).

$C_{14}H_{19}NO$	PM 217,307
Enantiomeric purity	96%
mp	260-261.5°C
$[\alpha]^{25}_D$	–73°
IR (KBr)	3419 cm^{-1} (O–H stretch) 3276 cm^{-1} (N–H stretch) 2930 cm^{-1} (C-H stretch) 1614-1578 cm^{-1} (C=C stretch) 1263 cm^{-1} (C-N stretch)
^{1}H NMR (DMSO)	0,658 δ (3H, d, CH_3), 1,073-1,135 δ (1H, m, CH), 1,183 δ (3H, s, CH_3), 1,388-1,673 δ (2H, m, CH_2), 2,258-2,563 δ (3H, m, CH_2 e CH), 2,824-2,960 δ (2H, m, CH_2), 6,420-6,473 δ (1H, m, Ph-*H*), 6,533-6,545 δ (1H, m, Ph-*H*), 6,765-6,806 δ (1H, m, Ph-*H*).
^{13}C NMR (DMSO)	δ 141.63, 136.60, 127.18, 126.06, 125.65, 125.49, 52.28, 42.77, 42.47, 39.08, 36.63, 33.31, 25.87, 13.98.

Table 3.2. (-)-*cis*-(*1R,5R,9R)-N*-normetazocine analytical data.

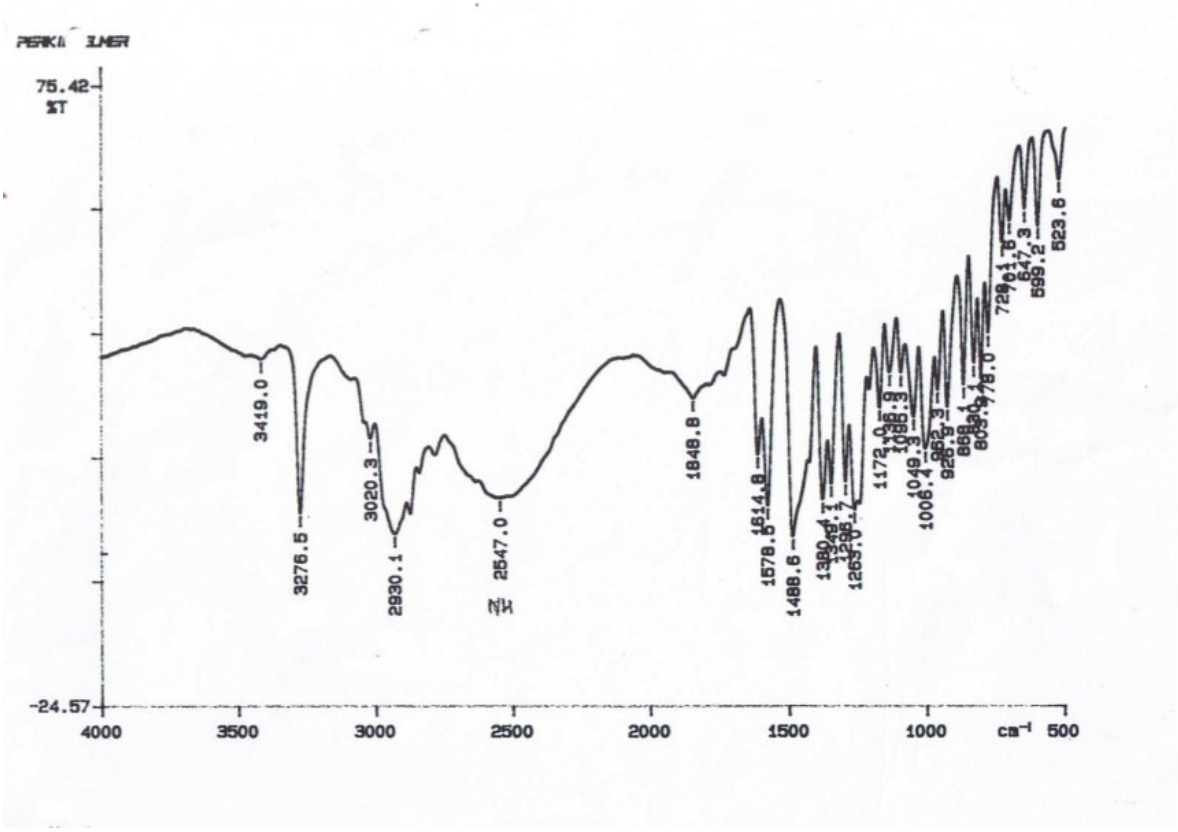


Figure. 3.4. (-)-*cis*-(*1R,5R,9R)-N*-normetazocine IR spectrum.

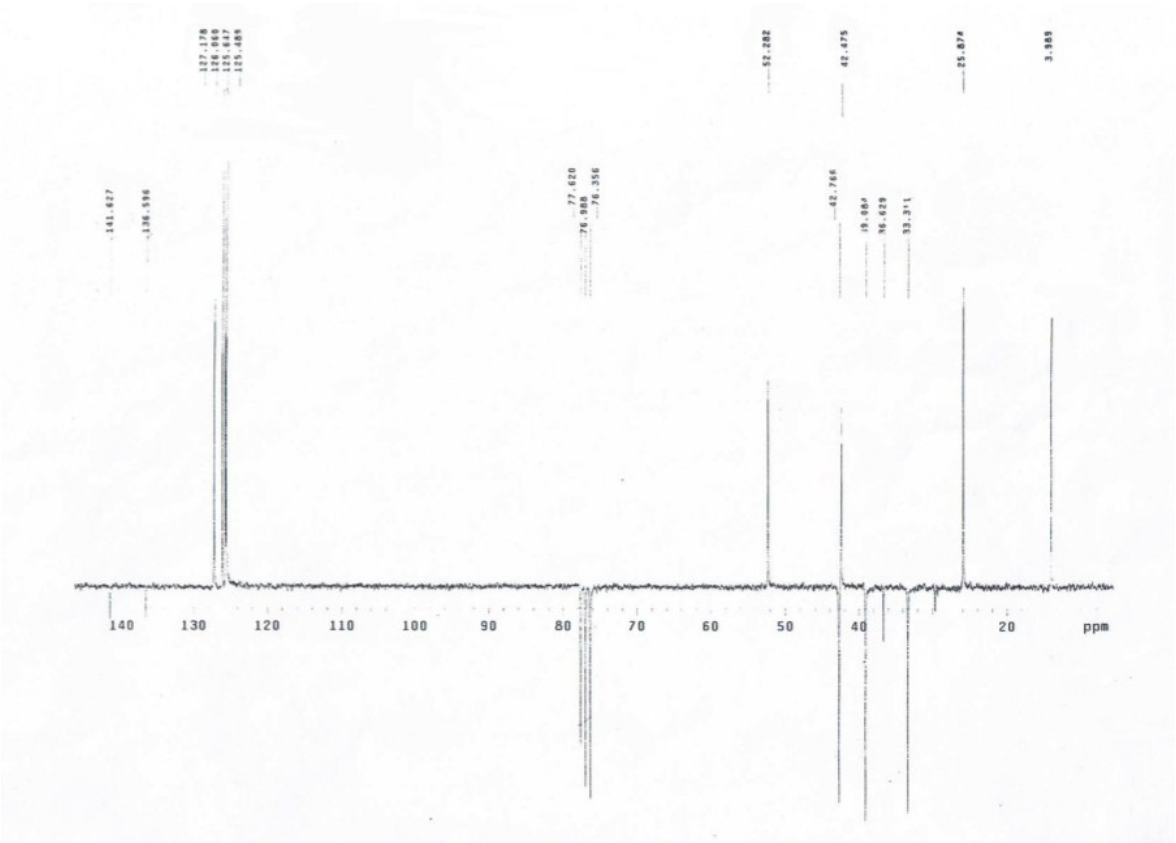
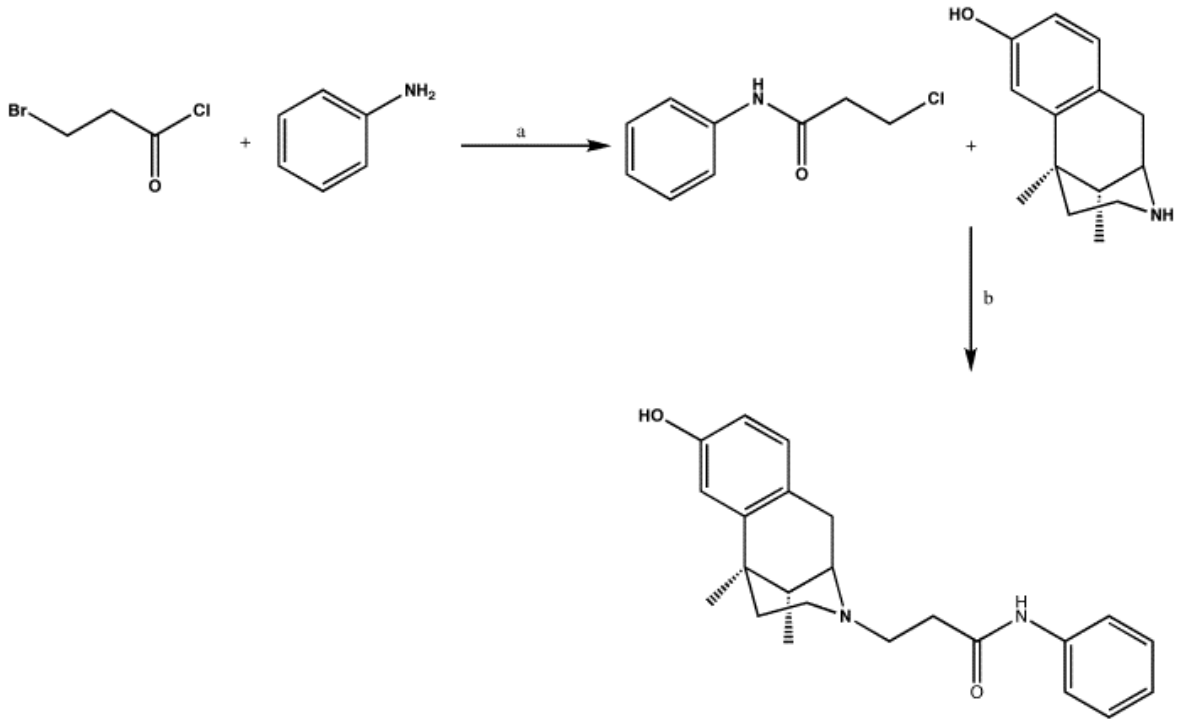


Figure. 3.5. (-)-*cis*-(*1R,5R,9R)-N*-normetazocine ^{13}C NMR spectrum.

LP1 synthesis

For LP1 synthesis (Scheme 3.2) was first made the intermediate 3-bromo-*N*-phenylpropanamide as reported by Pasquinucci et al. (2010). A mixture of *cis*-(-)-(*1R,5R,9R*)-N-normetazocine (2.3mmol), 3-bromo-N-phenylpropanamide (3.45 mmol), $NaHCO_3$ (3.45 mmol) and a catalytic amount of KI was stirred in DMF (10 ml) at 50 °C for 4 h. After cooling, the reaction mixture was diluted with AcOEt (200 ml) and H_2O 30 ml). The organic layer was separated, washed with brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* (Pasquinucci et al. 2010). The residue was purified by flash chromatography using $CHCl_3$ and CH_3OH as solvents. The analytical characterization of LP1 (Table 3.3) was performed using spectroscopy analysis ^{1}H-NMR, ^{13}C-NMR, IR and MS (Fig. 3.6, Fig. 3.7, Fig. 3.8).

Scheme 3.2. LP1 synthesis pathway



Reagent and conditions: a) DMAP, THF, 1h at 0°C and 3h at rt; b) *cis*-(-)-(*1R,5R,9R*)-*N*-normetazocine, $NaHCO_3$, KI, DMF, 20h at 50°C.

$C_{14}H_{19}NO$	PM 364,481
Yield	86%
mp	172-173°C
$[\alpha]^{25}_{D}$	–50° (c 1.0; MeOH)
IR (KBr)	1651 cm^{-1}(C=O stretch)
	3419 cm^{-1} (N–H stretch)
^{1}H NMR (DMSO)	δ11.27 (s, 1H), 7.58–7.54 (d, 2H), 7.38–7.26 (m, 2H), 7.13–7.10 (m, 1H), 6.97–6.94 (m, 1H), 6.78–6.76 (m, 1H), 6.68–6.62 (m, 1H), 3.07–2.53 (m, 6H), 2.51–2.49 (m, 2H), 2.27–2.13 (m, 1H), 1.97–1.77 (m, 2H), 1.44 (s, 3H), 1.46–1.36 (m, 1H), 0.88 (d, J = 5 Hz, 3H).
^{13}C NMR (DMSO)	δ 170.67, 154.95, 142.08, 138.53, 129.00, 128.32, 126.41, 123.88, 119.75, 113.51, 112.39, 57.79, 50.31, 44.73, 41.40, 36.14, 32.52, 29.51, 25.21, 23.60, 14.01
MS (MeOH)	*m/z* 365,2

Table 3.3. LP1 analytical data.

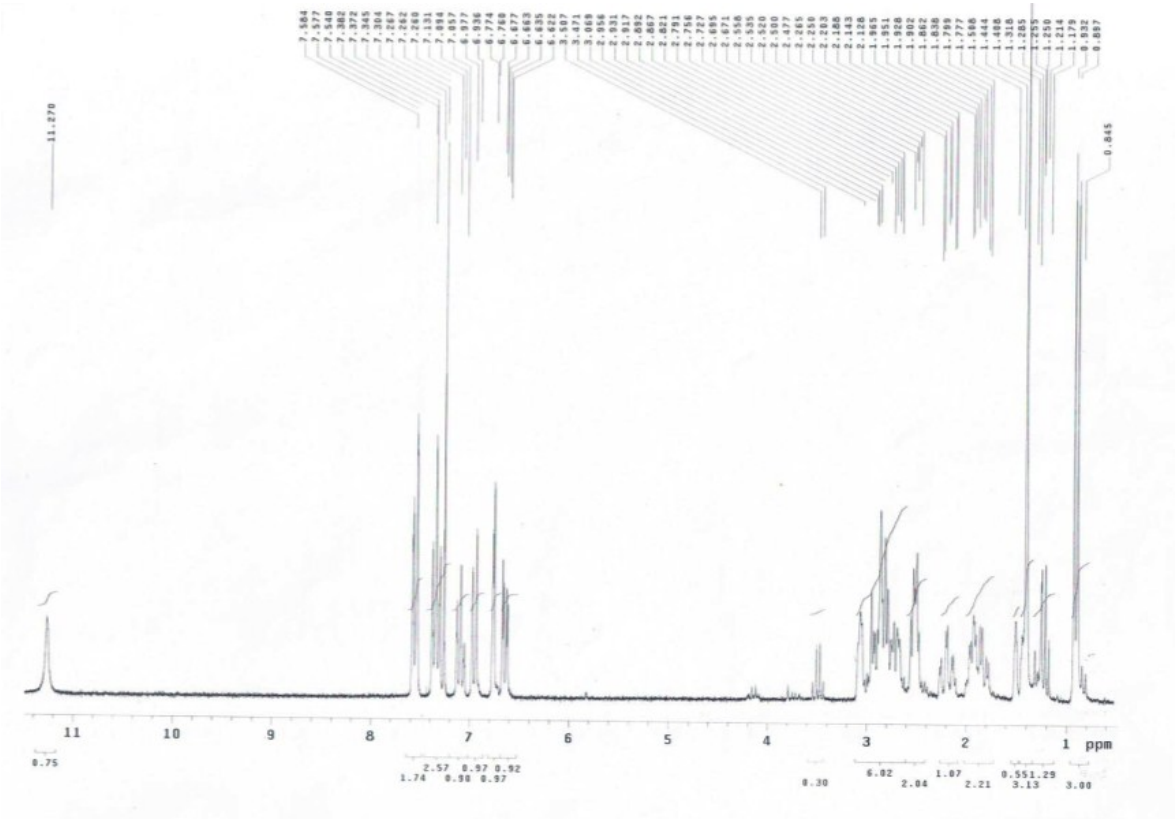


Figure. 3.6. LP1 ^{1}H NMR spectrum.

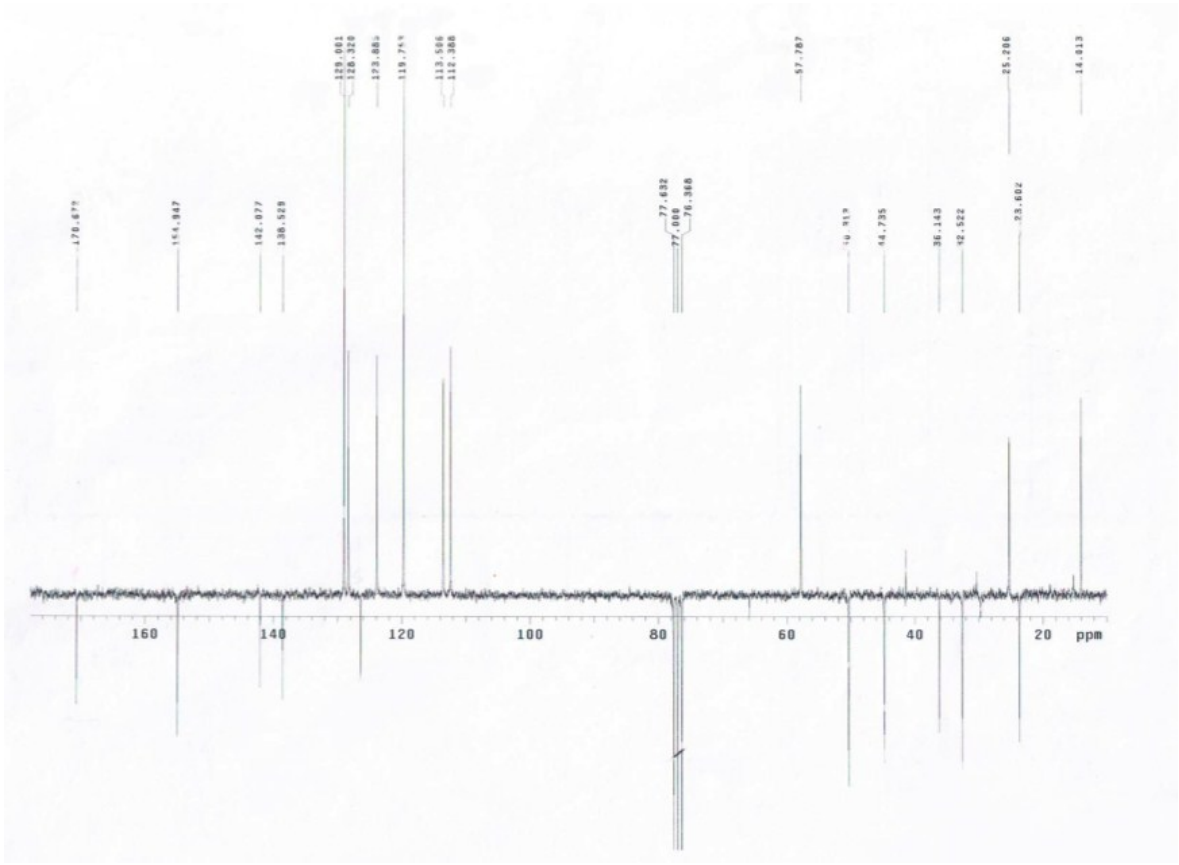


Figure. 3.7. LP1 ^{13}C NMR spectrum.

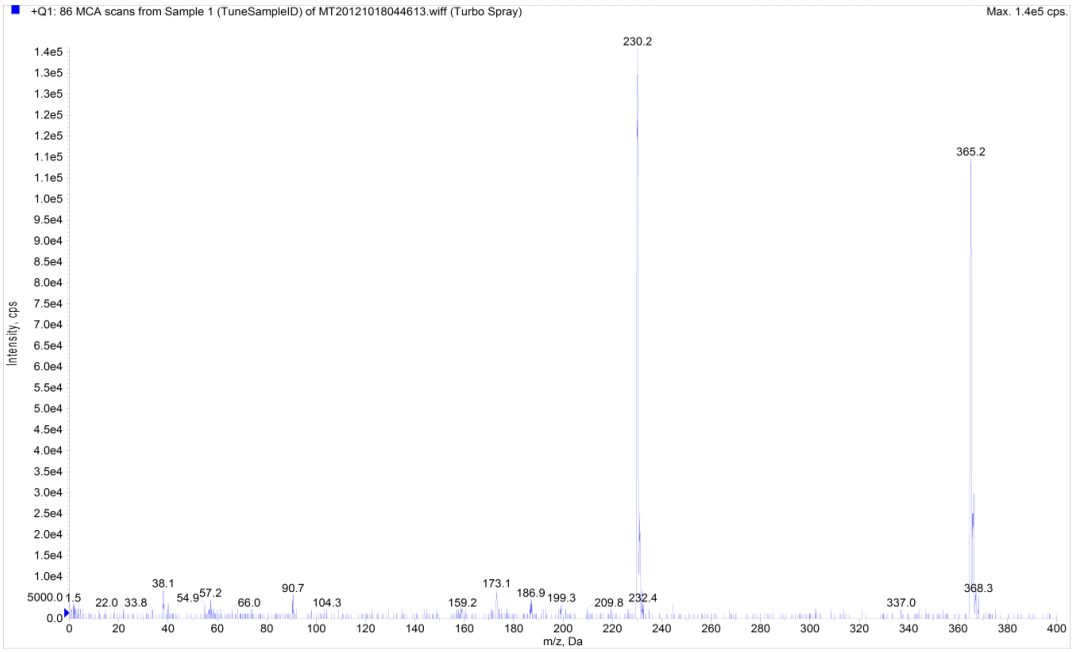


Figure 3.8. Mass spectra of LP1.

Cell Culture and Membrane Preparation

HEK293 cells stably expressing either the MOR or the DOR were grown in DMEM, supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin, at 37 °C under a 5% CO_2 atmosphere as previously described (Morou and Georgoussi 2005; Leontiadis et al. 2009). Confluent monolayers of HEK293 cells stably expressing the MOR or the DOR were harvested, collected by centrifugation at 1.500 rpm for 5 min and washed once with PBS (pH 7.5). Cell membranes were prepared as described by Georgoussi and Zioudrou (1993). Briefly, cell pellets were resuspended in ice-cold membrane buffer A (10 mM Tris-HCl, pH 7.5, and 0.1 mM EDTA), homogenized and centrifuged at 2 000 rpm for 3 min at 4 °C. Supernatants were further centrifuged at 45 000 rpm for 30 min at 4 °C. The membrane pellet was resuspended in ice-cold buffer A at a protein concentration of approximately 1 mg/ml and stored in aliquots at -70 °C. The protein concentration was determined according to the method of Bradford (1976).

[^{35}S]GTPγS Binding Studies

[^{35}S]GTPγS binding was performed on membranes from HEK293 cells stably expressing either the MOR or the DOR as described by Georgoussi et al. (1997). Membranes expressing the MOR (7.5 μg of protein per reaction) or the DOR (12 μg of protein per reaction) were added to a reaction mixture (100 μl) containing 20 mM HEPES (pH 7.4), 3 mM $MgCl_2$, 100 mM NaCl, 10 μM GDP, 0.2 mM ascorbate, 0.3-0.5 nM [^{35}S]GTPγS (50 nCi), and the appropriate ligand (0.1 nM-10 μM) and were incubated for

60 min at 30 °C or 4 °C for MOR and DOR, respectively. Non-specific binding was determined in the presence of 10 μM unlabelled GTPγS. The reaction was terminated by rapid filtration through GF/C Whatman filters followed by three washes with 4 ml of ice-cold 20 mM HEPES (pH 7.4) containing 3 mM $MgCl_2$ using a Brandel cell harvester. Bound radioactivity was measured by liquid scintillation counting (Liquid Scintillation Analyzer, Packard). Analysis of the binding data was performed using Origin 7.5 software (OriginLab Corporation, Northampton, USA). Data represent the percent of ligand-induced [^{35}S]GTPγS binding over basal activity, defined as [(specific binding/basal binding) × 100] – 100. Experiments were repeated at least three times and were performed in duplicate.

Drugs

DAMGO, DPDPE, GTP and all other reagents were of analytical grade from Sigma-Aldrich; [^{35}S]GTPγS (1250 Ci/mmol) was obtained from PerkinElmer; reagents for tissue culture were from Gibco and Invitrogen.

Results and Discussion

To characterise the efficacy of LP1 to stimulate [^{35}S]GTPγS binding were used membranes from HEK293 cells expressing either MOR or DOR (Pasquinucci et al. 2012). Treatment with LP1 produced a significant dose-dependent stimulation of [^{35}S]GTPγS binding in isolated cell membranes expressing MOR (Fig. 3.9). The EC_{50} value of LP1 to stimulate [^{35}S]GTPγS binding was lower than that of DAMGO. However, the maximal efficacy for [^{35}S]GTPγS binding produced by LP1 was lower than that measured for

DAMGO (Table 3.4). Similar measurements of LP1 effects on [^{35}S]GTPγS binding were performed in HEK293 cell membranes stably expressing the DOR. The maximal stimulation of [^{35}S]GTPγS binding by LP1 was similar to that detected for DPDPE. However, LP1 displayed a higher EC_{50} value than that detected with DPDPE (Table 3.4). At low concentrations of LP1, ranging from 1 nM to 100 nM, a dose-dependent inhibition of [^{35}S]GTPγS binding (35-40% at 100 nM) was observed (Fig. 3.9).

	EC_{50} (nM) ±SD[a]		E_{max}[b]	
	MOR	DOR	MOR	DOR
LP1	3.75±0.9	94.4±7	37±5.2	69±1.7
DAMGO	23±2.8	ND[c]	56±4	ND
DPDPE	ND	12.8±1.9	ND	66±3

[a]EC_{50} value is the concentration of compound needed to produce half maximal stimulation; [b]E_{max} value is the percentage of maximal stimulation; [c]ND, not determined.

Table 3.4. Measurements of [^{35}S]GTPγS binding.

This LP1 behaviour, depending on concentration, has led to the hypothesis that a ligand may achieve high affinity binding in several different ways, each having different effects on receptor activation (Meng et al. 2000).

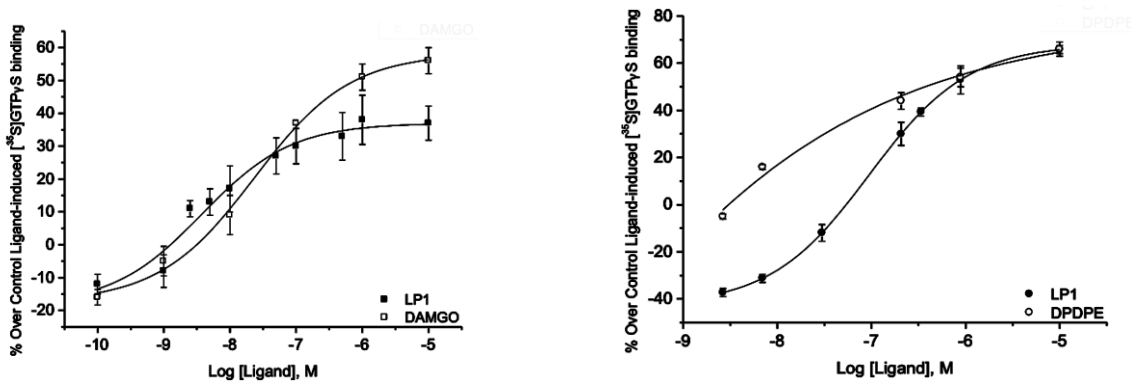


Figure 3.9. Effects of LP1 on [35S]GTPγS binding to membranes from HEK293 cells stably expressing the MOR (■). The ability of the MOR agonist DAMGO to stimulate the rate of guanine nucleotide exchange was also measured (□).Effects of LP1 on [35S]GTPγS binding to membranes from HEK293 cells stably expressing the DOR (●). The ability of the DOR agonist DPDPE to stimulate the rate of guanine nucleotide exchange was also measured (○).

The involvement of the different opioid receptor subtypes on LP1 antinociception was evaluated by tail flick test using selective MOR, DOR and KOR opioid antagonists LP1 (Parenti et al. 2012). NLZ, the selective MOR antagonist, at the dose of 35 mg/kg, administered 24 h prior to LP1, completely antagonized LP1 antinociception (the values recorded were significant at 40, 60 and 80 min (Fig. 3.10).

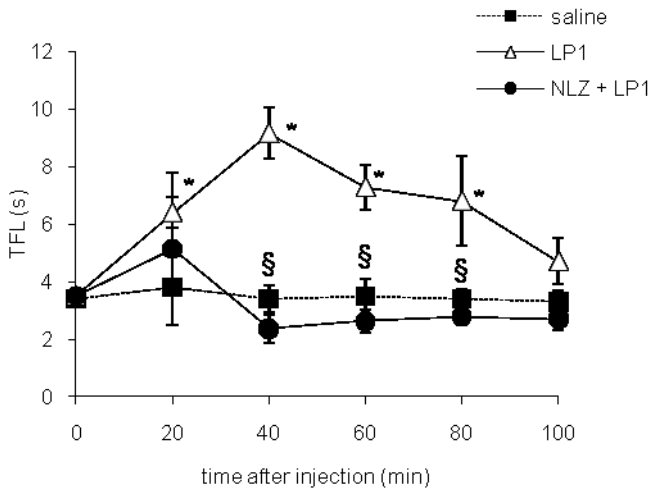


Figure 3.10. Effect of NLZ (35 mg/kg s.c.) on LP1 (3 mg/kg s.c.) antinociception.

Thus, a clear and unequivocal MOR involvement in the effect of LP1 was highlighted by the ability of the MOR selective antagonist NLZ to completely abolish the antinociceptive LP1 activity.

norBNI (10 mg/kg s.c.), a selective KOR antagonist, administered 30 min before of LP1, partially blocked the antinociceptive effect of the compound (Fig. 3.11).

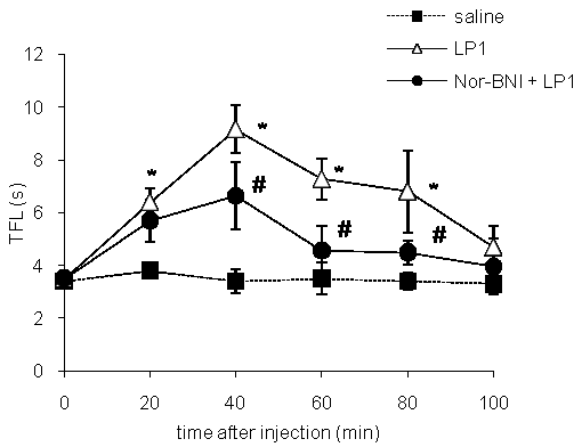


Figure 3.11. Effect of nor-BNI (10 mg/kg s.c.) on LP1 (3 mg/kg s.c.) antinociception.

However the involvement of KOR in the analgesic profile of LP1 was no supported by collected data first reported. In fact, as previously described, LP1 exhibited high and good affinity for MOR and DOR respectively, but a negligible affinity for KOR. In this regard, literature data reported that the antinociceptive effect of endomorphin-2, a known MOR agonist peptide with no significant affinity for KOR, was attenuated by norBNI pre-treatment (Horvath 2000; Ohsawa et al. 2001). It was demonstrated that MOR stimulation induces the release of endogenous dynorphins that, acting on KOR, elicit antinociception (Mizoguchi et al. 2006a, b). These findings prompted us to hypothesize that the contribution of KOR on LP1

antinociceptive activity may be an indirect phenomenon following MOR activation.

While, the selective DOR antagonist NTI, at the dose of 1 mg/kg s.c., 30 min before of LP1, did not modify the antinociceptive profile of LP1 (Fig. 3.12).

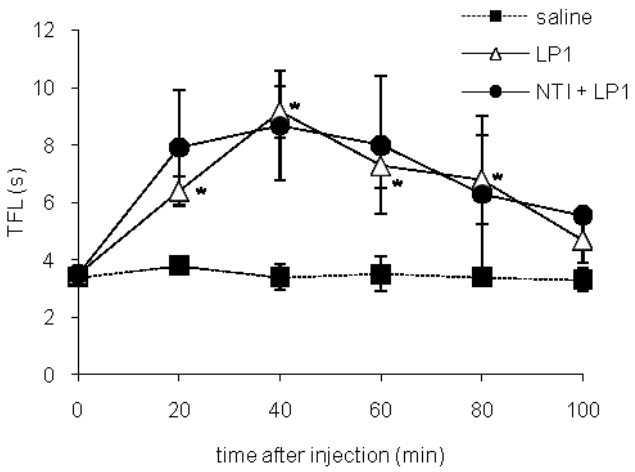


Figure 3.12. Effect of NTI (1 mg/kg s.c.) on LP1 (3 mg/kg s.c.) antinociception.

The DOR profile of LP1 (Parenti et al. 2012) was detected by exploring the ability of the compound to modify the antinociceptive effect of DPDPE, a selective DOR agonist. In this case, rats were first pre-treated with NLZ (35 mg/kg s.c.), and then LP1 (3 mg/kg s.c.) was administered prior to DPDPE (20 µg/5 µl i.c.v) abolishing the antinociceptive effect of the peptide (Fig. 3.13).

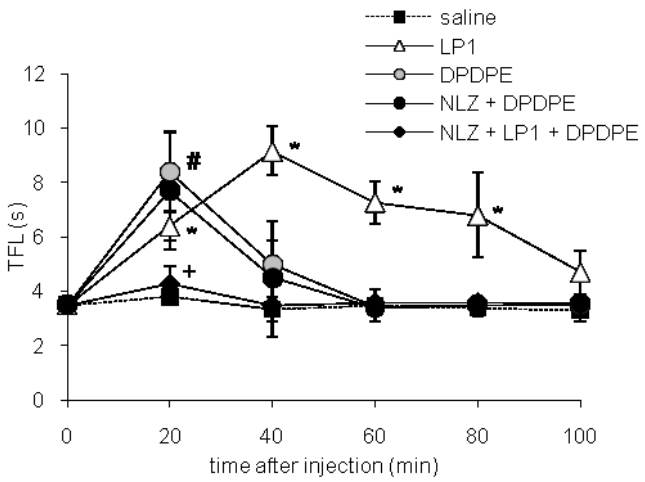


Figure 3.13. Effect of LP1 (3 mg/kg s.c.) on DPDPE (20 μg/5 μl/rat i.c.v.) antinociception in rats pre-treated with NLZ (35 mg/kg s.c.).

LP1-mediated antinociception was not modified by NTI, at least at the utilized dose, but in rats pre-treated with NLZ s.c. LP1 was able to attenuate significantly the antinociceptive effect induced by i.c.v. injection of the DOR agonist, DPDPE. Thus, these *In vivo* data seem to suggest that LP1 is a good MOR agonist also able to counteract the analgesia DPDPE-induced.

Moreover, to further delineate its pharmacological profile, the antinociceptive effect of LP1 was determined in NX-M pre-treated rats. The i.c.v. administration of NX-M, a quaternary derivative that does not readily cross the BBB, at the dose of 5 μg/5μl, 5 min before LP1, determined a significant reduction of the antinociceptive effect of the compound (Parenti et al. 2012). The tail flick latencies, instead, were not modified by the s.c. injection of NX-M when administered at the dose of 3 mg/kg, 30 min before of LP1 (Fig. 3.14).

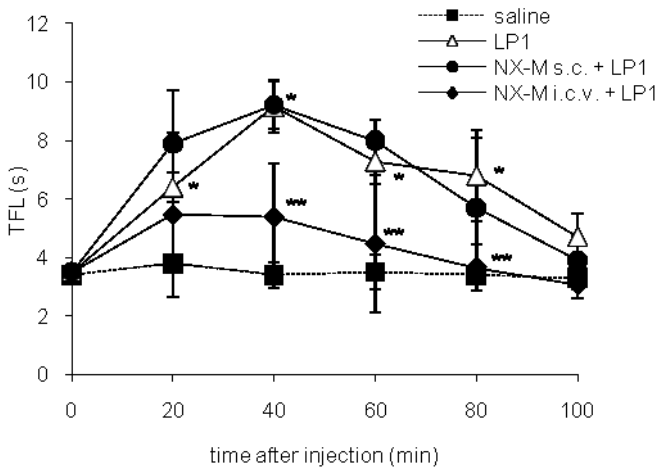


Figure 3.14. Effect of NX-M s.c. administered (3 mg/kg) and i.c.v. administered (5 μg/5 μl/rat) on LP1 (3 mg/kg s.c.) antinociception.

In rats pre-treated with s.c. NX-M, the LP1-evoked antinociception was not influenced. Conversely, i.c.v. NX-M pre-treatment led to a significant decrease of LP1 antinociceptive effect, demonstrating that the compound exerts its action predominantly in the CNS.

To define the LP1 tolerance-inducing capability, LP1 was evaluated at the dose of 4 mg/kg (s.c.) in comparison to morphine (10 mg/kg, s.c.) (Pasquinucci et al. 2012). The first injection of morphine and LP1 on the morning of day 1 induced a significant increase in tail flick latency. Rats receiving morphine twice per day showed on the third day of treatment a significant loss of antinociceptive effects. In contrast, LP1, which was administered under the same experimental protocol, maintained its antinociceptive profile until day 9 (Fig. 3.15).

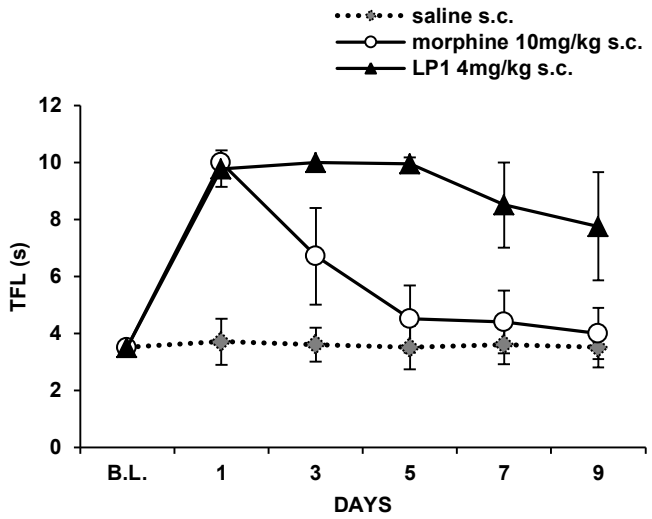


Figure 3.15. Effects of morphine (○) (n = 8) and LP1 (▲) (n = 10) on development of antinociceptive tolerance.

Tolerance to morphine-induced antinociception was observed on the third day of treatment. On the contrary, there was no diminution of LP1 antinociceptive effect until day 9 of observation. Thus, LP1 produced similar antinociceptive effects to morphine (Pasquinucci et al. 2010) with a less pronounced development of tolerance. Thus, LP1 may represent a novel pharmacological compound to alleviate pain without the induction of tolerance due to its ability to target simultaneously the MOR and DOR.

To evaluate LP1 behavioural effects an animal pain models of neuropathic pain induced by CCI was employed. CCI was produced according to Bennett and Xie (Bennett and Xie, 1988). The assessment of tactile allodynia consisted of measuring the withdrawal threshold of the hind paw in response to probing with a series of calibrated von Frey's filaments (Scoto et al., 2009). Allodynic threshold in CCI rats displayed a decrease which reached a significant value of 2.5 ± 1.4g 14 days after surgery (Fig. 3.16, panel A) in respect to the contralateral, non-lesioned paw (11.9 ± 1.2

g). LP1 was tested s.c. in ligated animals at the 14th days after surgery (Fig. 3.16, panel B) at 3 mg/kg, dose extrapolated as the lowest effective dose from dose-response curve in acute experiments (Pasquinucci et al., 2010).

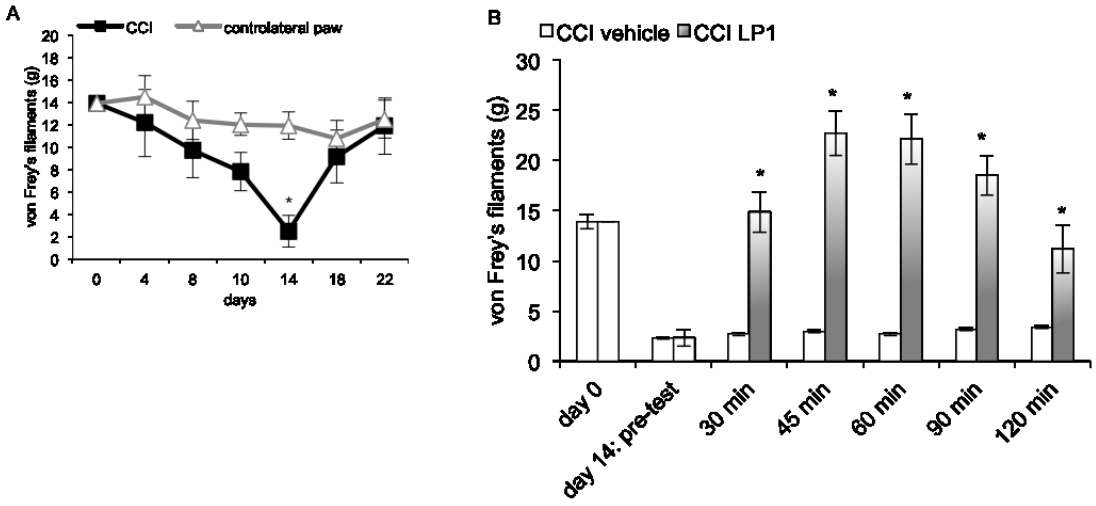


Figure 3.16. (A) Time-course (days) of CCI-induced allodynia measured with Von Frey's filaments.(B) Effect of LP1 (3 mg/kg s.c.) in CCI rats on mechanical allodynia measured with Von Frey's filaments.

It was registered antiallodynic values significantly different from the vehicle from 30 until 120 min from LP1 administration with the highest effect at 45 min (22.6± 2.2) and 60 min (22.1 ± 2.5) after s.c. administration (Parenti et al., submitted). For the effect on thermal hyperalgesia (Fig. 3.17 panel A), LP1 was administered 8 days after surgery. The opioid ligand caused an increase of the thermal thresholds that were significantly enhanced at 30, 45, 60, 90, 120 min, (Fig. 3.17 panel B) relatively to vehicle-treated CCI rats.

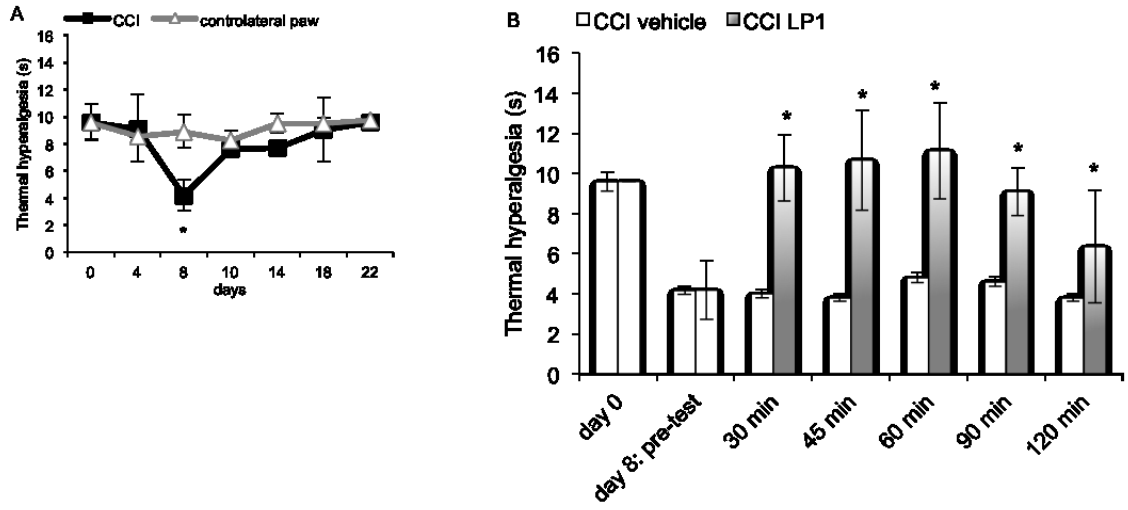


Figure 3.17. (A) Time-course (days) of CCI-induced hyperalgesia measured with Plantar test. (B) Effect of LP1 (3 mg/kg s.c.) in CCI rats on thermal hyperalgesia measured with Plantar test.

Effects of LP1 were also evaluated in rats that had undergone carrageenan inflammation in the left paw. In Figure 3.18 panel A and 3.20 panel A it was highlighted the time course of carrageenan treated rats over the 6 h testing period. Following inflammatory injury, animals developed progressive behavioural signs of mechanical and thermal sensitization.

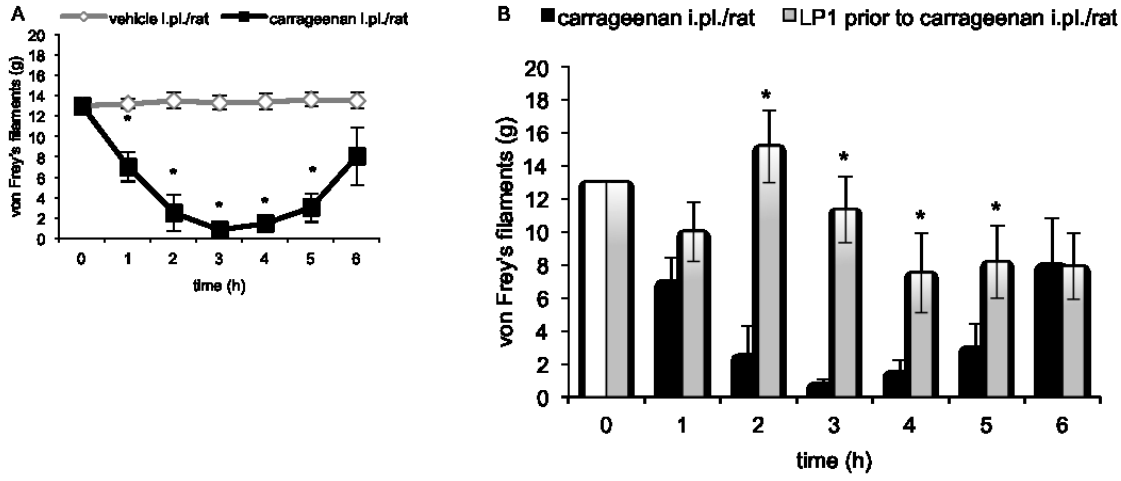


Figure 3.18. (A) Time-course (hours) of carrageenan-induced allodynia measured with Von Frey's filaments (B) Effect of LP1 (3 mg/kg s.c.),injected 15 min before i.pl. carrageenan on mechanical allodynia measured with Von Frey's filaments.

LP1 given s.c., at the dose of 3 mg/kg immediately prior to i.pl. carrageenan, caused a rise in the allodynic threshold values, significantly preventing the development of the allodynic effect induced by the inflammatory agent from 2 h to 5 h post-treatment (Fig. 3.18 panel B). LP1, given s.c. at the dose of 3mg/kg immediately prior to i.pl. carrageenan (Fig. 3.19 panel B), determined a significant increase of the thermal threshold values from 2 h to 5 h post-treatment respect to the thermal hyperalgesic values carrageenan-induced.

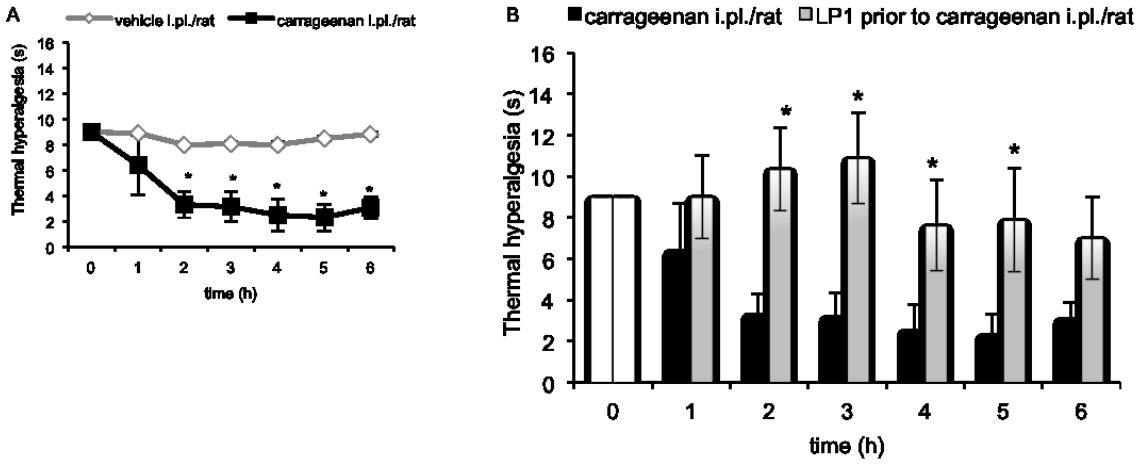


Figure 3.19. (A) Time-course (hours) of carrageenan-induced hyperalgesia measured with Plantar test (B) Effect of LP1 (3 mg/kg s.c.), injected 15 min before i.pl. carrageenan on thermal hyperalgesia measured with Plantar test.

The LP1 administration produced a reduction of pain behaviours (allodynia and hyperalgesia) either in a model of neuropathic pain, developed after sciatic nerve ligation, and in a model of inflammatory pain, induced by i.pl. injection of carrageenan. This is a promising finding since LP1 was found to be as effective in acute nociceptive as in neuropathic and inflammatory pain models.

Conclusion

The MOR agonist-DOR antagonist ligand LP1 is a central acting antinociceptive agent. In addition to be a valid antinociceptive for acute pain, LP1 could be a suitable drug candidate for the management of pain persistent conditions requiring long-term therapy, because of its low potential to induce tolerance. *

* **ABBREVIATIONS**: MOR, μ-opioid receptor; DOR, δ-opioid receptor; KOR, κ-opioid receptor; LP1, 3-[(2R,6R,11R)-8-hydroxy-6,11-dimethyl-1,4,5,6-tetrahydro-2,6-methano-3-benzazocin-3(2H)-yl]-N-phenylpropanamide; CNS, central nervous system; NLZ, naloxonazine; NTI, naltrindole; norBNI, norBinaltorphimine; NX-M, naloxone methiodide; i.c.v. intracerebroventricular; s.c., subcutaneous; CCI, chronic constriction injury; DMF, dimethylformamide; AcOEt, ethyl acetate; NMR, nuclear magnetic resonance; IR, infrared spectroscopy; MS, mass spectroscopy; PBS, phosphate-buffered saline; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; DAMGO, H-Tyr-**D**-Ala-Gly-***N***-MePhe-Gly-OH; DPDPE, [D-Pen^2,D-Pen^5]-Enkephalin; BBB, blood brain barrier; TLC, thin layer chromatography; i.pl., intraplantar.

Chapter 4

DESIGN AND SYNTHESIS OF CONFORMATIONALLY CONSTRAINED COMPOUNDS AS NEW TRAMADOL-LIKE CANDIDATES

Design and synthesis of conformationally constrained compounds as new tramadol-like candidates

Background

Chronic pain is one of the most prevalent, costly, and disabling conditions in both clinical practice and the workplace. It is treated with many drug classes, such as narcotic analgesics, anticonvulsants, antidepressants and topical anaesthetics, but with a limited cost-benefit profile (Argoff 2011). Despite opioid analgesics are very effective against nociceptive pain, they may have an unsatisfactory therapeutic window for chronic pain treatment due to the development of analgesic tolerance and to other debilitating adverse effects (Brush 2012). Considering the multitude of pain transmission and modulatory pathways, the multitarget approach could represent a rational strategy to overcome limits associated with analgesics acting at a single target. For instance, multitarget ligands, with an opioid and non-opioid mechanism of action, showed a favourable and safety clinical profile in neuropathic pain conditions requiring long-term management (Argoff 2011). In particular, combining MOR agonism with monoamine reuptake inhibition is a valid approach to improve the therapeutic range of opioids (Bannister et al. 2009). In fact, the different and complementary mechanisms of action may additively or synergistically enhance the analgesic efficacy and/or attenuate side effects of MOR agonists by reducing the requirement for MOR activation. In this context, Tramadol - an atypical, racemic opioid - combines weak MOR activation with inhibition of 5-HT and NE reuptake. The combination of

these complementary mechanisms of action results in a significant analgesic activity. Tramadol was chosen as *lead compound* to design and synthesis of new structural analogues.

Tramadol: clinical relevance

Tramadol hydrochloride (Raffa and Friderichs, 2003) (Fig. 4.1) is a synthetic opioid from the aminocyclohexanol group and produces its analgesic effect through central mechanisms.

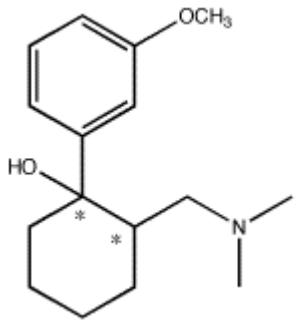


Figure 4.1. Tramadol structure.

In fact, Tramadol is an analgesic with opioid agonist properties also able to act on the neurotransmission of NE and 5-HT. Respect to other opioid analgesics of comparable efficacy and NSAIDs, Tramadol is of particular interest because of the relative lack of serious side effects (Leppert 2009). For instance, in comparison with typical opioid agonists, such as morphine, pethidine and the partial agonist buprenorphine, Tramadol rarely causes respiratory depression (Moore and McQuay, 1997; Ossowska and Wolfarth, 1994) or physical dependence. Tramadol was developed by the German pharmaceutical company Grünenthal in the early 1960s and nowadays marketed as Tramal®, Ultram®, Contramal®, Tridol®, Trodon®, Adolonta®, Top-Algic® and Nobligan® (Bamigbade and Langford, 1998).

The management of postoperative pain is the primarily clinical application of Tramadol where it proved to be equivalent or superior to commonly prescribed oral analgesic combinations with a good safety profile because lack of respiratory depression, tolerance, and constipating effects (Vickers et al. 1992; Osipova et al. 1991; Wilder-Smith et al. 1994).

Tramadol is used in the field of oral surgery for the relief of dental extraction pain. A clinical study demonstrated that Tramadol, in comparison with NSAIDs - like paracetamol or ketorolac - and combination analgesics - such as propoxyphene and codeine - is equivalent or more effective. Although Tramadol is an effective analgesic in pain after dental extraction, in patients with chronic dental pain related to chronic periodontitis and chronic pulpitis Tramadol is no indicated (Moroz et al. 1991). In this pain state, the efficacy was improved by adding NSAIDs. On the contrary, Tramadol 50 mg three times per day may be an optimal chance for those patients who are unable to take NSAIDs, which are currently first line analgesics after dento-alveolar surgery. In fact, in patients that underwent dento-alveolar surgery Tramadol proved to be a useful analgesic. Moreover, Tramadol may be safely combined with non-opioid analgesics, especially with paracetamol, with an improvement in analgesia but no increasing toxicity. A combined preparation of Tramadol (37.5 mg) and paracetamol (325 mg) is now available (Bamigbade and Langford, 1998).

Several studies have shown Tramadol to be effective against pain related arthritis. In patients in whom NSAIDs no provide adequate analgesia for chronic degenerative disease of the hip and/or knee, Tramadol and

dextropropoxyphene reduced significantly these pain syndromes. For both medications were reported a high incidence of mostly mild adverse events. By this clinical study emerged that Tramadol was superior to dextropropoxyphene for symptom relief and that the higher incidence of fatal overdoses were reported for dextropropoxyphene, making Tramadol a safer long-term alternative. Tramadol was also compared with diclofenac sodium for the treatment of painful hip or knee osteoarthritis. No difference was found in treatment efficacy, activities of daily living and global patient preference. Although no serious adverse events were reported for both analgesics, there were significantly more side effects associated to Tramadol respect to diclofenac sodium. Moreover, while the administration of Tramadol 100 mg and ketorolac 30 mg as single therapeutic agents led, without significative difference, an adequate analgesia, the co-administration of Tramadol 50 mg and ketorolac 30 mg resulted to a very good analgesia coupled to a low incidence of side effects such as nausea and sweating (Bianchi et al. 1999).

In trauma and in acute or chronic musculoskeletal problems Tramadol is also effective. In fact different clinical trials proved that in patients with musculoskeletal injuries the i.v. administration of 100 mg Tramadol produce a satisfactory analgesia (Berghold et al. 1991; Mattia and Coluzzi 2005). However, in some orthopedic cases single oral doses of Tramadol (50 or 100 mg) resulted unsatisfactory to relief pain because Tramadol, being absorbed from the small intestine, gives its effect after 90 minutes. Contrarily, in the same pain condition Tramadol i.v. administered at the dose of 200-250 mg relieved pain. Tramadol is also used for the pain

treatment in the early postoperative period after coronary artery bypass graft surgery administered at a dose of 1 mg/kg i.v. over 20 minutes. However, in a percentage of patients Tramadol can cause negative inotropic effects (Bamigbade and Langford, 1998). As proved by clinical investigations, in which were compared infusions of alfentanil at 12.5 μg/kg per hour with infusions of Tramadol up to 600 mg in 24 hours, for both drugs pain scores were the same, as well as time to extubation, respiratory variables, nausea and vomiting but significantly less shivering was noted with Tramadol. Tramadol is used effectively for the cessation of postoperative shivering. In a study Tramadol 1 mg/kg administered i.v. was compared favorably with pethidine 0.3 mg/kg for this indication. The effects on post-anesthetic shivering were potentiated by physostigmine.

In a clinical study relative to postoperative analgesia in a group of 20 patients undergoing thoracotomy, Tramadol i.v. administered was compared with epidural morphine. Both analgesics were similar to relief postsurgical pain with minimal differences in respiratory function.

In neurosurgery field and in particular in patients with head injuries, Tramadol was used but in susceptible individuals it may cause convulsions by increasing levels of CNS catecholamines. In neurosurgical patients after craniotomy Tramadol was compared with codeine phosphate. Tramadol 50 mg may be superior to codeine phosphate with regard to pain scores and supplementary analgesic requirements and in these patients convulsions were no observed.

Tramadol is also used in children over the age of 12 years and in many countries in children over the age of one year. In children with moderate

to severe postoperative pain Tramadol 2 mg/kg i.m. administered produced an excellent pain relief. Comparably to nalbuphine, children with postoperative pain following urological surgery were pain free within one hour of the initial dose. The most frequent adverse effects were fatigue, nausea and vomiting, while haemodynamic and respiratory adverse effects were not observed.

In patients with acute myocardial infarction and unstable angina Tramadol, administered as an i.v. bolus injection, produced sufficient analgesia although it can develop respiratory distress. However, Tramadol does not seem to be the best option for these patients, in whom the use of traditional opioid analgesics is well recognized (Bamigbade and Langford, 1998).

Tramadol seems to be efficacious in a wide range of cancer pain syndromes for pain related to bony secondary and least effective in neurogenic pain (Leppert 2009). The WHO suggested tramadol as belonging on step 2 of analgesic ladder, this mean that after non-opioid analgesics alone fail, Tramadol in combination with non-opioid analgesics could be effective (Grond et al. 1992). A comparison of tramadol with morphine showed that, despite an effective pain relief could be obtained with Tramadol, morphine was more effective in severe cancer pain. Moreover, respect to morphine Tramadol induces less tolerance. Over the eight-week period, there was an average 7% increase in Tramadol dose compared with a 41% increase in the morphine dose. The adverse events profile, as the severity of nausea and constipation, was significantly better with Tramadol. A higher initial dose should be used to improve pain

control with Tramadol, but the high pain score could also reflect Tramadol's slower onset of action. Tramadol may be particularly useful for patients who are more sensitive to the adverse effects of strong opioid analgesics (e.g., sedation, fatigue, constipation). In fact, Tramadol may be considered as an alternative to small doses of strong opioids such as morphine, oxycodone, hydromorphone, transdermal fentanyl or transdermal buprenorphine (Sunshine et al. 1992).

Tramadol exhibited an antiallodynic effect in a model of neuropathic pain in rats (tight ligation of the L5 and L6 nerve roots of the sciatic nerve distal to the dorsal root ganglion), and this effect is only partially antagonized by naloxone. Tramadol has recently been reported to be effective for the pain of diabetic neuropathy in a multicenter randomized, placebo-controlled double blind study (O'Connor and Dworkin, 2009).

Pharmacokinetic properties

Tramadol is available in several formulations and routes of administration such as capsules (50 mg), ampoules (100 mg), and as dispersible (50 mg) and sustained release tablets (100, 150 and 200 mg) (Barkin 2008; Guidelines for the assessment and management of chronic pain, WMJ 2004). It is licensed for oral, i.m. and i.v. use in adults and in children over the age of 12 years. Tramadol is administered orally at a dose of 50 – 100 mg three to four times daily to a recommended maximum of 400 mg. The sustained release tablets allow twice-daily administration instead of the usual three or four times daily intake required for the immediate release formulations. The onset of action is between 20 and 40 minutes

after administration of the capsules and 60 minutes after the sustained release tablets.

For oral administration Tramadol bioavailability is 70%, higher than that quoted for morphine (15–65%). The peak plasma concentration after orally administered Tramadol is reached between two hours for capsules, and five hours for sustained release tablets. Tramadol undergoes first pass process and binding to plasma proteins is in the region of 20%. Tramadol crosses the placenta and only 0.1% of the dose passes into breast milk (Klotz and Fischer-Bosch 2003).

Tramadol is also available as oral drops and 20 drops equivalent to 50 mg, and as rectal suppositories a 100 mg suppository can be administered up to four times daily. The bioavailability of Tramadol suppositories was 80% and the time to reach a serum concentration associated with analgesic efficacy was approximately 90 min (Liao 1992). The maximum serum concentration was achieved after two to four hours (Lee 1993).

Tramadol for parenteral administration is available as 100 mg in 2 ml ampoules. By i.m. or i.v. routes the usual dose is 50–100 mg. Moreover it is available Tramadol s.c., epidural and i.a.

Adverse effects

Tramadol adverse events are typically opioid in nature, however it is important to note that respiratory depression does not occur at therapeutic doses (Delikan and Vijayan 1993). Most common adverse effects following acute, short-term multiple dose of Tramadol are nausea, tiredness/fatigue, vomiting, sweating, drowsiness and postural hypotension. Tramadol, may induce seizures, particularly in the presence

of other pro-convulsant drugs. Convulsions can occur after its parenteral or oral administration and when doses exceed. Factors that increase the risk of convulsions are preexisting epilepsy or the concomitant use of TCAs and SSRIs. Tramadol, as opioid analgesic, produces urinary retention although this effect may occur less frequently than with some other opioids such as morphine. The drug is a myocardial depressant at supratherapeutic dose and may induce hypotension and orthostatic hypotension. Tramadol, like other opioids, produces muscle rigidity by stimulating striatal MOR. The unique feature of Tramadol is its lack of respiratory depression when administered within the therapeutic range, and when compared with the traditional opioids, making it especially suitable for use in the postoperative period. Tramadol's weak opioid activity confers enhanced safety, in particular a low potential for the development of tolerance, dependence or abuse.

Tramadol: the importance of the mechanism of action

Despite Tramadol is structurally related to morphine (Fig. 4.2) and is a weak opioid agonist, clinical experience and pharmaco-epidemiological data indicated a profile that is not typical of an opioid because of its low potential to induce respiratory depression, abuse and psychological dependence.

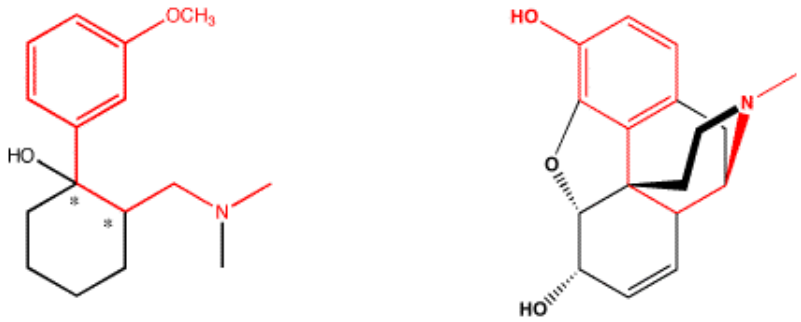


Figure 4.2. Common structural points of morphine (right) and tramadol (left).

These favorable features are strongly related to the peculiar mechanism of action of Tramadol. The original compound was a *cis–trans* mixture that was easily separated by solubility differences. The pharmacological testing of the individual enantiomers (Gillen et al. 2000) showed that the *cis*-isomers were a stronger analgesic. Radioligand-binding experiments performed on Tramadol displayed only a modest affinity *versus* opioid receptors. Specifically, Tramadol possessed a weak affinity for the MOR and even weaker affinity for DOR and KOR. As shown in Table 4.1, in comparison with codeine, dextropropoxyphene and morphine, the Tramadol MOR affinity was 10, 60 and 6,000 times less, respectively

	K_i (μM)		
Compound	MOR	DOR	KOR
Morphine	0.00034	0.092	0.66
d-propoxyphene	0.034	0.38	1.22
Codeine	0.2	5.1	6.0
Tramadol	2.1	57.6	42.7

Table 4.1. Comparison of K_i values between Tramadol and typical opioid analgesics.

The low binding affinity made it very unlikely that Tramadol itself was responsible for the opioid component of its mechanism of action. It was speculated that similarly to compound codeine, which is metabolically converted to morphine, Tramadol might be demethylated to the active metabolite O-desmethyl Tramadol responsible for analgesia. In fact, Tramadol is metabolized by the cytochrome P450 enzyme system in the liver to form five metabolites (M1 to M5) from phase I reactions (N- and O-demethylation), and six metabolites from phase II conjugation reactions

(forming glucuronides and sulphates of M1, M4 and M5) (Lintz 1982; García-Quetglas et al. 2007; Wu et al. 2002). CYP2D6 is the enzyme responsible for O-demethylation to mono-O-desmethyltramadol known as M1 (Fig. 4.3), which is the only pharmacologically active metabolite. It was undertaken to radioligand binding experiments by which emerged for the M1 metabolite a higher affinity *versus* MOR than Tramadol, in fact M1 was about 300-fold more potent (Gillen et al. 2000).

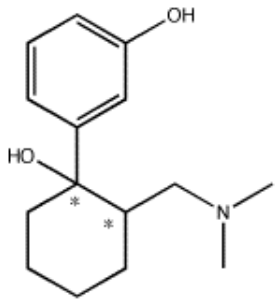


Figure 4.3. M1-metabolite

However, the metabolic biotrasformation of Tramadol into the active metabolite M1 was no enough satisfactory to support the analgesic effect of the drug. In fact, as demonstrated in animal pain models, the antinociceptive effect of Tramadol was partly resistant to inhibition by the opioid antagonist NX. This finding suggested the involvement of a non-opioid component in the mechanism of action of Tramadol (Raffa et al. 1992). After a number of experimental studies (Hui-Chen et al. 2004) was clearly established the involvement of a non-opioid component in the analgesic effect Tramadol-induced due to its ability to inhibits the neuronal reuptake of 5-HT and NE (Berrocoso et al. 2006). In *in vitro* experiments, the NE uptake blocker, cocaine, prevented the NE effect of Tramadol as well as the uptake blocker 6-nitroquipazine abolished the 5-HT effect. Furthermore, the uptake inhibition of 5-HT and NE takes place

at the same range of concentrations (0.5–50 μM) that cause MOR activation, suggesting that both mechanisms are active. In *in vivo* studies performed in rats was demonstrated that the antinociception Tramadol-elicited was partially reversed by yohimbine, an α2- adrenoceptor antagonist, and the 5-HT antagonist, ritanserin (Raffa 1993). Similar results have been demonstrated in human volunteers.

Tramadol is marketed as a racemic mixture of the (+)-enantiomer (*1R,2R*)-2-[(dimethylamino)-methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride and the (-)-enantiomer (*1S,2S*)-2-[(dimethylamino)-methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride (Fig. 4.4). Both Tramadol enantiomers contribute to the analgesic effect in different way. In fact, the (+)-enantiomer inhibits 5-HT reuptake more than the (–)- enantiomer and causes an increase in 5-HT efflux. Conversely, the (–) enantiomer inhibits NE reuptake more than the (+)-enantiomer and causes increased stimulation-evoked release by presynaptic autoreceptor activation (Table 4.2) (Friderichs et al. 1992).

	K_i(μM)		
Compound	MOR	NA	5-HT
Morphine	0.00034	-	-
(*R*,*R*)-Tramadol	1.3	2.51	0.53
(*S*,*S*)-Tramadol	24.8	0,43	2.35
(1*RS*,2*RS*)-Tramadolo	2.1	0.78	0.9
(*R*,*R*)-M1	0.0034		
(*S*,*S*)-M1	2.4		
(1*RS*,2*RS*)-M1	0.0054		

Table4.2. K_i values of Tramadol, Tramadol enantiomers, M1 and M1 enantiomers.

Analogously to Tramadol, the resulting M1 metabolite exists as mixture of (+)- and (-)-enantiomers (Fig 4.4). M1 exerts stereoselective effects *in vitro* because the (+)-M1 activates the MOR, while (–)-M1 has a weaker opioid and norepinephrinergic component.

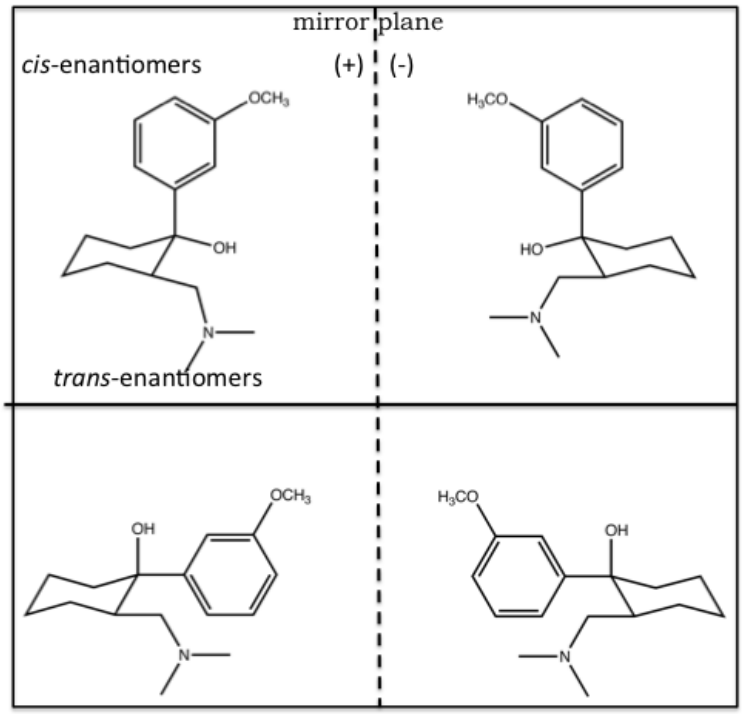


Figure 4.4. Enantiomers of tramadol.

By [^{35}S]GTPγS binding studies were highlighted great differences between (±)-Tramadol and its (±)-M1 metabolite. (+)-M1 and (-)-M1 stimulated GTPγS binding whereas (±)-Tramadol did not do so. Since the maximal GTPγS binding achieved with (+)-M1 and (-)-M1 was below that obtained with DAMGO, the M1 enantiomers behave as partial agonists compared to DAMGO. The most potent compound (+)-M1 (EC_{50}=0.86±0.21 μM) showed the highest efficacy (E_{max}=52±2%).

In the tail flick test i.v. application of the (+)-M1 metabolite established this enantiomer as the major opioid component of Tramadol (Kögel et al. 1999) because of its strong analgesic effect. To further confirm the important role of the M1 metabolite for the opioid component, the analgesic effect of Tramadol was evaluated in extensive *versus* poor metabolizers of CYP2D6, since the cytochrome P450 is involved in the O-demethylation (Collart et al. 1993b; Poulsen et al. 1996). Decreased M1 levels reported in poor metabolizers coincide with a decreased analgesic effect of Tramadol. The (+)- and (-)-enantiomers individually produce centrally mediated (spinal) antinociception in mice. The different contribution of each enantiomer of Tramadol and its M1 metabolite in the analgesic profile is synergistic. This means that the racemic drug is more efficacious. In fact, the measured ED_{50} of (±)-Tramadol is lower than the theoretical value calculated if the contribution of the enantiomers were simply additive. Thus combined as a raceme the enantiomers of Tramadol interact in a synergistic manner. Importantly, it was proved that the synergistic interaction was no reflected in the adverse effects profile of the drug. In fact, adverse effects predominate in one or other of the enantiomers and, in part, they antagonize each other. The enantiomers interact less than synergistically or even less than additively in several preclinical tests predictive of clinical side effect liability, such as inhibition of colonic propulsive motility, impairment of rotarod performance, respiratory rate, and blood pressure.

Other mechanisms of action

Medei et al. (2011) investigated the effects on the L-type Ca^{2+} currents of rat ventricular myocytes of Tramadol and its enantiomers, evaluating the negative cardiac inotropic action. By this investigation was found that the effectiveness of each Tramadol enantiomers to inhibit L-type Ca^{2+} currents was twice greater than that observed with Tramadol raceme and such effect seems unrelated to the activation of opioid receptors. The inhibition of L-type Ca^{2+} currents induced by Tramadol and its enantiomers could explain, at least in part, their negative cardiac inotropic effect.

Minami et al. (2011) investigated the effects of M1 on SPR expressed in Xenopus oocytes by examining SP-induced Ca^{2+}-activated Cl^- currents. In fact, SPR belongs to the family of Gq protein–coupled receptors that activate the PKC and Ca^{2+}-mobilization by stimulation of phospholipase C. In this study, M1 metabolite inhibited the SPR-induced Cl^- currents at pharmacologically relevant concentrations. These findings might help to elucidate the pharmacological basis of M1 metabolite and Tramadol and provide a better understanding of their neuronal action and antinociceptive effect. Moreover, the inhibitory effects of M1 on SPR might also contribute to the Tramadol side effects.

Drug design process

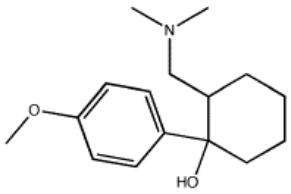
Tramadol - an atypical, racemic opioid - combines weak MOR activation with inhibition of 5-HT and NE reuptake (Sevcik et al. 1998). As previously described, both enantiomers of Tramadol and M1 metabolite are characterised by different mechanisms of action. Thus, this means that the contribution of the different mechanisms of action to the analgesic

profile changes over time. In fact, as the parent molecule is metabolized, the contribution of 5-HT and NE reuptake inhibition is reduced while the contribution of MOR agonism is increased, resulting in a complex time- and metabolism-dependent pattern of pharmacological activities. Given this background and considering that an improved pain relief remains a primary need in chronic pain management, some Tramadol-like compounds were designed and synthesized. To this aim, extensive SAR studies on Tramadol and relative derivatives were performed. Thus, pharmacophoric features and their critical distances were highlighted in order to identify a model that represented the interaction with MOR of these series of compounds.

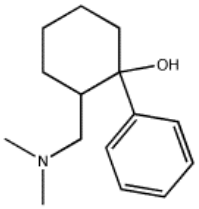
SAR studies

To design new Tramadol-like derivatives were performed extensive SAR studies on Tramadol analogues by which it was possible to screen the pharmacophoric elements crucial for the interaction with MOR.

Meta position of the substituent in phenyl ring is crucial for the interaction with MOR. In fact, the *para* and *orto* positions are no well tolerated. As reported in the Patent n° 3,652,589, for the derivative bearing the $–OCH_3$ group in *para* position (1), the analgesic activity measured in mice was significantly lower (ED_{50}= 100 mg/kg accompanied by convulsions) than the parental compound Tramadol (ED_{50}= 11,2 mg/kg). The critical importance of the aromatic *meta* substituent was further demonstrated by the synthesis of a Tramadol analogue lacking of this substituent (2). In *in vivo* assessment performed in mice no analgesic activity was reported.

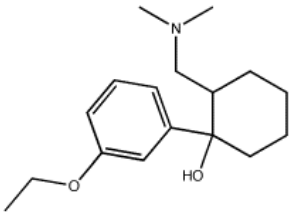


1

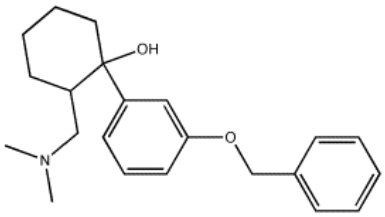


2

The replacement of the *meta*-ether group with -OH increased the affinity and efficacy *versus* MOR, as well as the replacement with an ester group. By replacing the *m*–OCH_3 with the *m*–OH, which occurs during metabolic conversion of Tramadol, MOR affinity is increased by two orders of magnitude (Raffa and Friderichs, 2003). Analogues 3a-3b, bearing superior homologous of –OCH_3 (e.g., ethoxy, propoxy, phenoxy, or benzyloxyl), had low affinity for MOR (3b ED_{50}= 30 mg/kg).

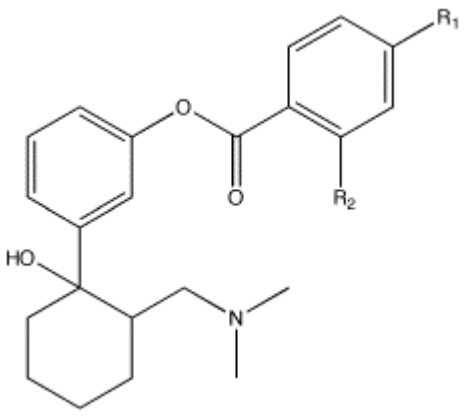


3a



3b

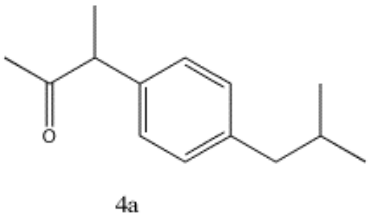
These compounds were metabolically desalkylated to the same O-hydroxy derivative as for Tramadol, but compared with this last, the higher homologues showed an increased toxicity. In several patents was reported as favourable the replacement of the *m*–OCH_3 group with ester function (4). In the hot plate test, compounds 4a-4e displayed an improved antinociceptive profile (Table 4.2) (Patent n°WO 00/27799, 2000).



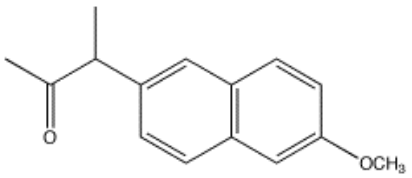
4

Compound	% incresing time of
4a	568
4b	539
4c	416
4d	546
4e	327
Tramadol	**218**

Table 4.3.

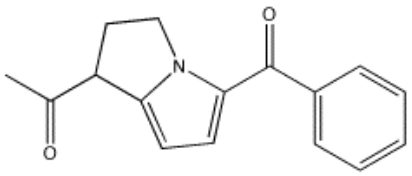


4a

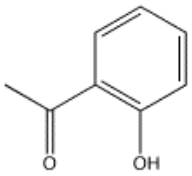


4b

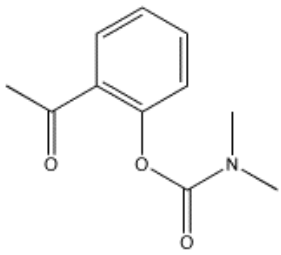
R=



4c

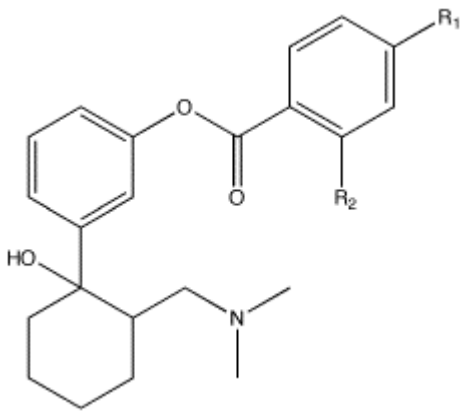


4d



4e

Another study (Patent n° US 2003/0232891) explored the influence of polar substituents on the aromatic ring of the *meta* substituent (5).



5

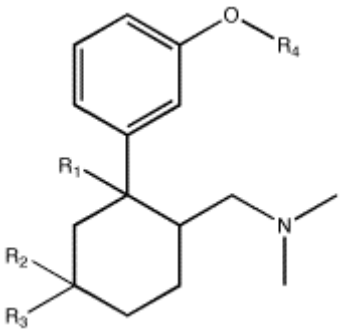
R_1	**R_2**	**% increasing time of answer**
CF_3	OAc	710
CF_3	OH	724
Cl	OH	400
Me	OH	525
Ome	OH	688
NO_2	OH	661
Tramadol		**218**

Table 4.4.

As shown in the Table 4.4, in comparison with Tramadol the resulting compounds exhibited an improved antinociceptive effect assessed by hot plate test. Unfavourable for the efficacy on MOR is the substitution of the *meta*-ether group with halogens.

In contrast to modifications at the aromatic ring, substitution of –OH of the cyclohexyl by halogen or even H had only a weak influence on opioid receptor affinity, as splitting-off the –OH and introducing a 1,2- or 1,6-double bond (6).

For instance, in Table are reported ED_{50} values expressed as mg/kg obtained by tail flick test after i.v. administration (Table 4.5). In comparison to Tramadol, an increased antinociceptive activity was found for analogues with structure 6.

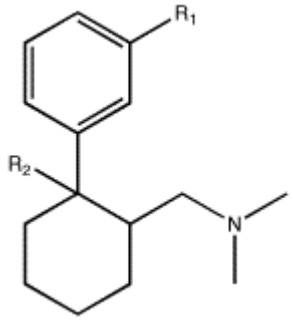


6

R_1	R_2	R_3	R_4	ED_{50}
F	H	H	H	2,28
Cl	H	H	H	2,78
H	H	H	Me	10,7
H	H	H	H	1,13
H	H	H	$COCH(Me)_2$	4,61
OH	OH	H	Me	3,93
OH	OH	H	OH	7,34
Tramadol				**13,6**

Table 4.5.

Patent n° C07C 255/59 reported Tramadol derivatives in which the hydroxyl group was alkylated (7) (Shao et al. 2008). Obtained compounds displayed an increased affinity *versus* MOR, but their affinity *versus* the NE and 5-HT transporters is reduced for alkyl group superior to the ethyl group (Table 4.6).

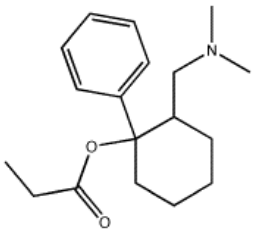


7

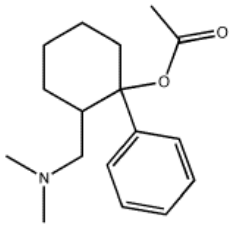
Compound	Ki (nM) MOR	Ki (nM) NA	Ki (nM) 5-HT
R_1= OH R_2= Me	56	525	10000
R_1= OH R_2= Et	130	3776	1424
Tramadol	7600	3861	1493

Table 4.6.

Esterification of the hydroxyl group linked to cyclohexyle is a negative modification (8a-8b) (Patent n° 3,652,589).

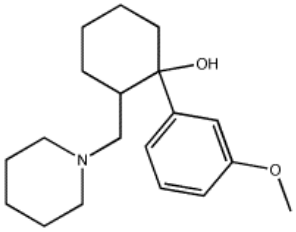


8a

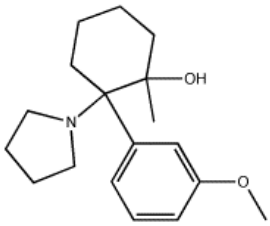


8b

By tail flick test in mice, compounds 8a and 8b, respect to Tramadol exhibited a strong reduction of the antinociceptive potency (ED_{50}= 50 mg/kg for both compounds). Substitution of -CH_3 of the basic N with higher homologues led to an unfavourable modification because hider the binding to MOR. The same occurred incorporating the N into a five- or six-membered ring system (9a-9b) (compound 9a ED_{50}=175 mg/kg).

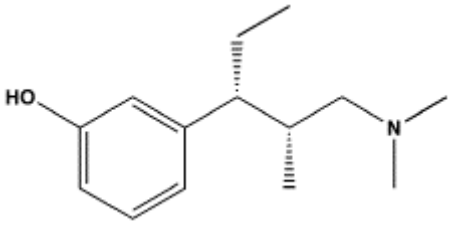


9a



9b

A newest Tramadol derivative is Tapentadol (10) a centrally acting oral analgesic approved by the US Food and Drug Administration in November 2008 for the treatment of moderate to severe acute pain.



10

It is a novel MOR agonist (K_i= 0.1μM; relative efficacy compared with morphine 88% in a [^{35}S]GTPγS binding assay) and NE reuptake inhibitor. Tapentadol exhibited analgesic effects in a wide range of animal models of acute and chronic pain (hot plate, tail flick, writhing, Randall Selitto, mustard oil colitis, CCI, and SNL), with ED_{50} values ranging from 8.2 to 13 mg/kg after i.p. administration in rats. Tolerance development to the analgesic effect of Tapentadol was twice as slow as that of morphine.

Design

The pharmacophoric features of Tramadol - represented by an aromatic ring linked to a quaternary carbon, a two-carbons chain and an amino group - were maintained in the structure of the series of compounds designed (Fig. 4.5).

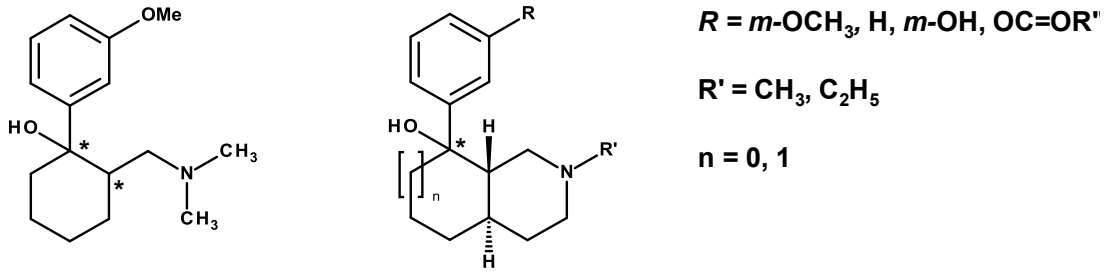
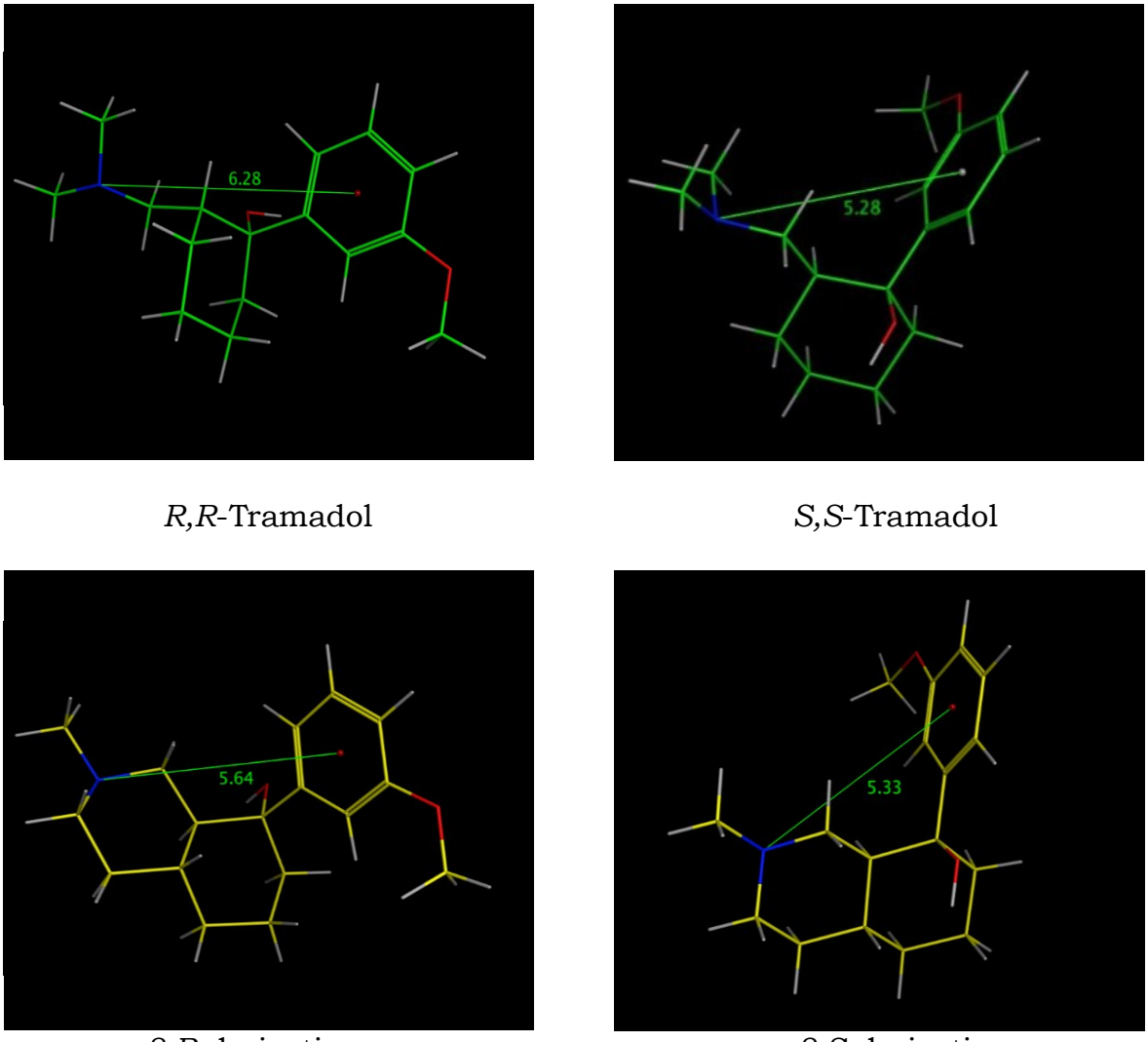


Figure 4.5. Structure of new Tramadol-like derivatives.

It has been designed and synthesised a series of conformationally constrained compounds as new Tramadol-like candidates, in which two

pharmacophoric elements of Tramadol - the lateral chain and the basic nitrogen - are constrained in a cyclised structures represented by the *trans*-decahydroisoquinoline and octahydro-1*H*-cyclopenta[c]pyridine nuclei. The conformationally constrained nuclei may exert a positive role by blocking the final compounds in a semi-rigid conformation that could enhance the MOR efficacy.

To design the new Tramadol-like derivatives, besides the pharmacophoric features, were also considered their relative distances.



R,R-Tramadol

S,S-Tramadol

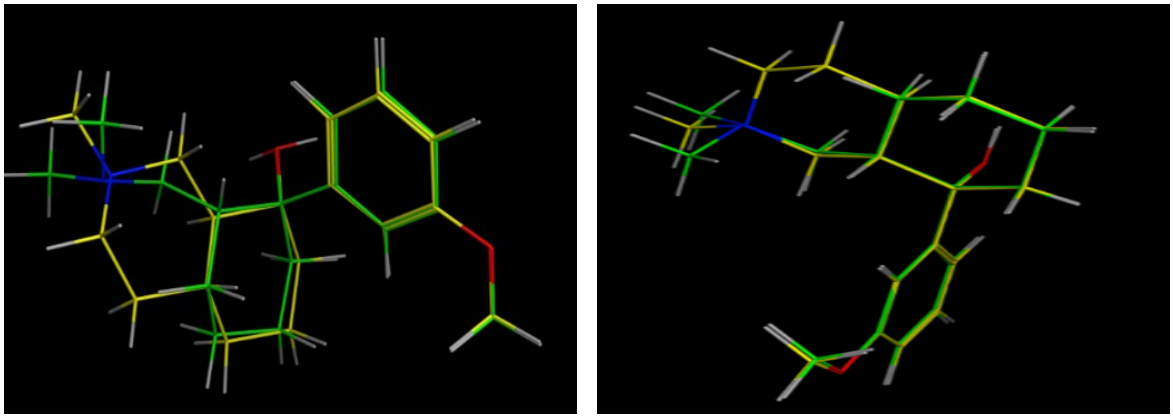
8 R-derivative

8 S-derivative

Figure 4.6. Distances between basic N and aromatic ring in Tramadol enantiomers and in enantiomers with *trans*-decahydroisoquinoline nucleus.

To this aim, for both enantiomers of Tramadol and enantiomers of the new Tramadol-like compounds with the *trans*-decahydroisoquinoline nucleus, the distances between the basic nitrogen and the aromatic ring were detected. As shown in Fig. 4.6 the distances revealed are significantly comparable.

Moreover, both enantiomers of the *trans*-decahydroisoquinoline derivatives and the enantiomers of Tramadol were undertaken to superimposition studies using MOE by Chemical Computing Group. For this study, minimized compounds were aligned rigidly by the common basic nitrogen (Fig. 4.7).



R,R-Tramadol/8*R*-derivative *S,S*-Tramadol/8*S*-derivative

Figure 4.7. Superimposition analysis

A reasonable superimposition of pharmacophoric elements of (+)-enantiomer of Tramadol and the new compound with 8*R* stereochemistry was detected – as well as a reasonable superimposition was revealed for the (-)-enantiomer of Tramadol with the new 8*S*-compound. This analysis further highlighted that the semi-rigid structure of the trans-decahydroisoquinoline nucleus constrain the aromatic moiety, respect to

the basic nitrogen, to assume distances comparable to that of Tramadol enantiomers. Collectively, superimposition data could expect a similar mechanism of action of 8*R-trans*-decahydroisoquinoline derivative with (+)-(*R*)-enantiomer of Tramadol, and 8*S-trans* derivative with (-)-(*S*)-enantiomer of Tramadol. Thus, *in vivo* analgesic profile of racemic mixture of new synthesized compounds could be similar to activity of (±)-Tramadol. Reference data reported that the distinct profiles of the two Tramadol enantiomers combine to produce effective analgesia within wider therapeutic range. Thus, its ED_{50} value subtends a synergic interaction between its multiple modes of action.

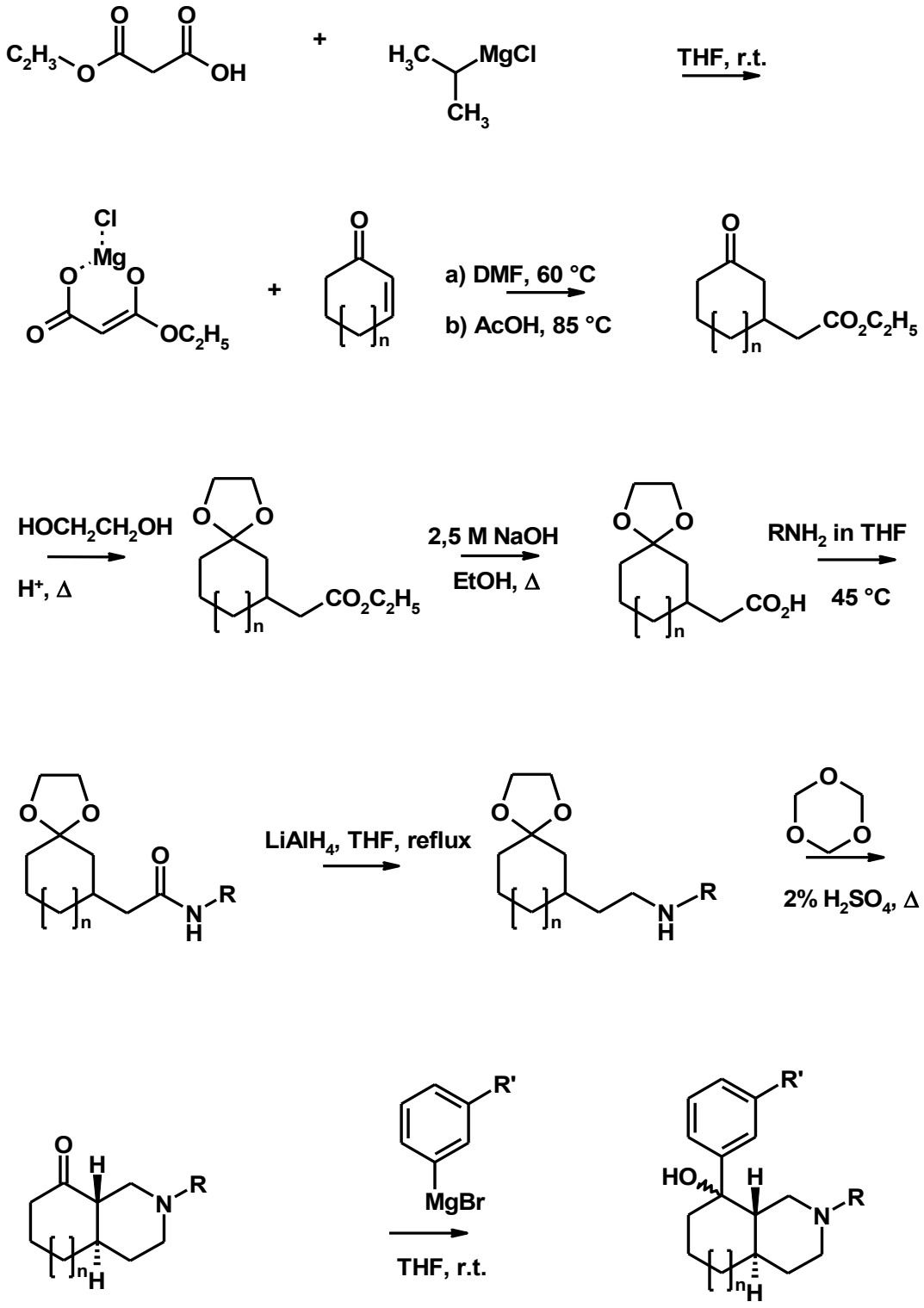
Experimental Procedure

Synthesis of final compound is reported in the scheme 4.1 and 4.2.

Conformationally constrained nuclei synthesis has provided a five-stage approach (Simoni et al 2005). One-pot synthesis was used to obtain first intermediates (Mcmurry et al. 1978). Briefly, in a representative procedure, magnesium monoethyl malonate underwent Michael addition to 2-cyclohexenone or 2-cyclopentanone and the resulting malonate monoethyl ester intermediates were decarboxylated by McMurry's procedure. Protection of the relative ketones as ethylene acetal (Babler et al. 1978) allowed their subsequent conversion into amides via the acid intermediate (Reimann et al. 2004). Their successive reduction to amines was performed in about 90% yield with $LiAlH_4$ in THF and resulting amines were undertaken to a Mannich-type cyclization to obtain *trans*-decahydroisoquinoline and octahydro-1*H*-cyclopenta[c]pyridine nuclei

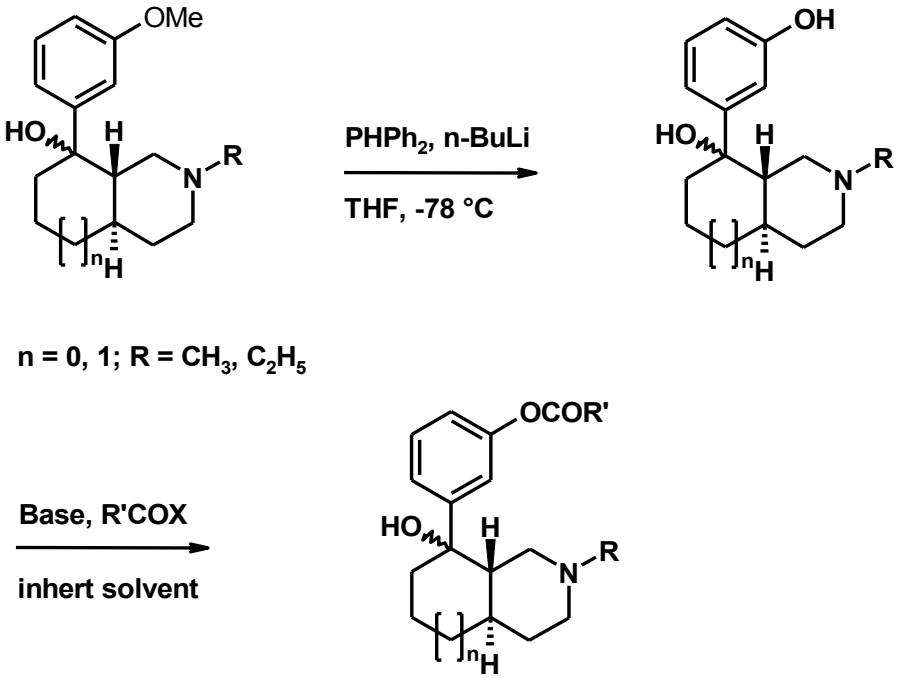
(Simoni et al 2005). A further synthetic step was required to obtain some final compounds by using appropriate Grignard reagents (Patent US 3,652,589).

Scheme 4.1. Synthesis pathway of compounds



n = 0, 1; R = CH_3, C_2H_5; R' = *m*-OCH_3, H

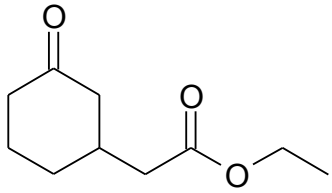
Scheme 4.2. Synthesis pathway of compounds



Chemistry

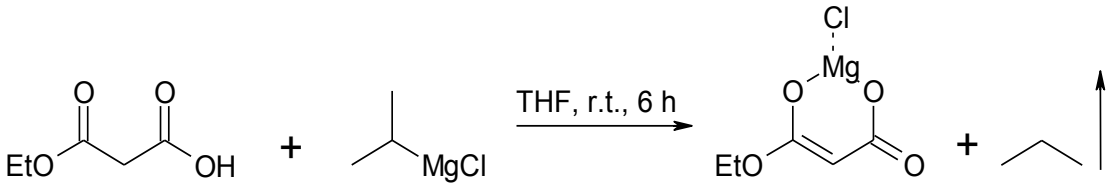
All compounds were characterised by IR Perkin Elmer FT-IR 1600; ^{1}H- and ^{13}C-NMR Varian Inova-200 MHz and 500 MHz in $CDCl_3$ with TMS as internal reference at 25°C; MS AB sciex, API2000, ESI. TLC was performed in aluminium sheets Kieselgel 60 F254 (Merck). Flash chromatography was performed using silica gel 60 (0,063-0,2 mm) Merck.

Synthesis of ethyl-2-(3-oxyciclohexyl)ethanoate



$C_{10}H_{16}O_3$ Mw 184,232

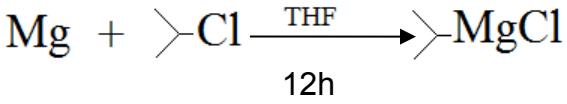
One-pot synthesis was performed to obtain first intermediates. The process required three steps. The first step consists in the synthesis of the magnesium mono-ethyl malonate.



Under anhydrous conditions 77,52 mmol of i-PrMgCl *, ** 2 M solution in THF diluted in 36 ml of THF were added dropwise to 38 mmol of mono-ethyl malonate. The resulting propane development caused an increasing of temperature until 60-70°C. The mixture was stirred at rt until the complete development of propane.

** Synthesis of isopropyl magnesium chloride*

Under a stream of nitrogen, magnesium turnings (110 mmol) was covered by a small amount of THF, and a solution of isopropyl chloride (100 mmol) in THF (50 ml) was added dropwise, keeping the temperature of the mixture below 30 °C (water bath). After the addition was completed, the reaction mixture was stirred for 12 h at rt. The grey solution of i-PrMgCl was titrated prior to use.

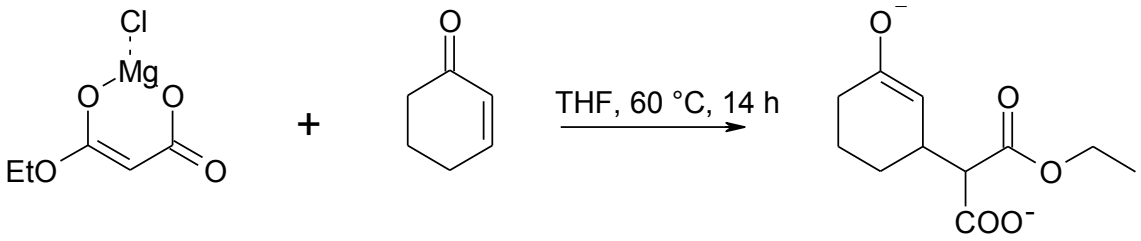


*** Isopropyl magnesium chloride titration*

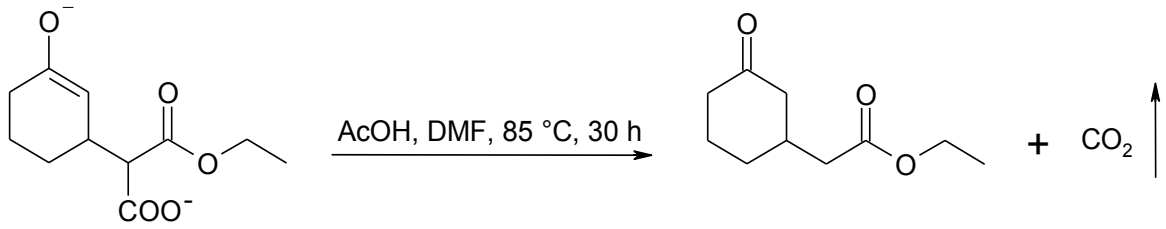
The titration was performed using as titrating agent I_2 in a saturated solution of LiCl.

$$RMgCl + I_2 \xrightarrow{\text{soluzione 2,5 M di LiCl in THF}} I\text{-} + MgCl_2 \cdot LiCl$$

For the saturated solution, LiCl (100 mmol) was previous dried under vacuum at 140°C for 4 hours. After cooling at rt, 200 ml anhydrous THF were added and the resulting suspension was stirred for 24 hours at rt until LiCl was completely dissolved. To 1 mmol of I_2 were added 5 ml of saturated solution LiCl. The resulting brown solution was cooled at 0°C and the i-PrMgCl reagent was added dropwise until the colour disappeared. The amount of i-PrMgCl consumed contain 1 equivalent of Grignard reagent relative to I_2.



In the second step magnesium monoethyl malonate was dissolved in 70 ml DMF dry under a nitrogen atmosphere and cyclohexenone or cyclopentenone (38 mmol) was added via syringe. The solution was warmed to 60°C for 12 hours.

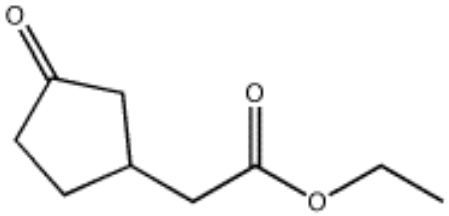


Decarboxylation was the last step. After cooling to rt AcOH (12.7 mmol) was added to the mixture. After further heating to 85" for 30 hours to effect decarboxylation, the solution was cooled, diluted with water, and extracted three times with ether. The organic layers were washed with saturated bicarbonate and brine, then dried over Na_2SO_4 and concentrated to yield first intermediates (89%).

$C_{10}H_{16}O_3$	PM 184,232
Yield	89%
TLC	CH_2Cl_2
	Rf 0,41
IR *neat*	3456 cm^{-1} (C=O *stretch overtone*, br)
	2939.5 cm^{-1} (C-H *stretch*)
	1728.3 cm^{-1} (C=O *stretch*)
	1713.6 cm^{-1} (C=O *stretch*)
	1225.8 cm^{-1} (C-C(O)-C *bend*)
	1157-1034 cm^{-1} (C-O *stretch*)

^{1}H NMR ($CDCl_3$)	δ 1,28 (3H, t, CH_3, *J*= 6.8 Hz), 1,36-1,80 (4H, m, CH_2), 2,08-2,18 (6H, m, CH_2), 2,25-2.32 (1H, m, CH), 4,09-4.19 (2H, q, CH_2, *J*= 7 Hz).
^{13}C NMR ($CDCl_3$)	δ 14,14 (CH_3), 24,71 (CH_2), 30,78 (CH_2), 35,50 (CH), 40,91 (CH_2), 40,99 (CH_2), 47,34 (CH_2), 60,41 (CH_2), 171,71 (OC=O), 210,50 (C=O).

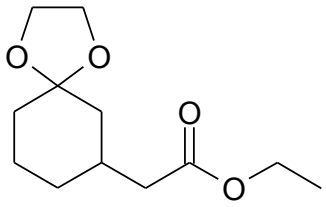
Synthesis of ethyl-2-(3-oxyciclopentyl)ethanoate



$C_9H_{14}O_3$ Mw 170,206

$C_9H_{14}O_3$	PM 170,206
Yield	80%
TLC	CH_2Cl_2
	Rf 0,43
IR *neat*	3466 cm^{-1} (C=O *stretch overtone*, br)
	2929.5 cm^{-1} (C-H *stretch*)
	1725.3 cm^{-1} (C=O *stretch*)
	1715.6 cm^{-1} (C=O *stretch*)
	1225.8 cm^{-1} (C-C(O)-C *bend*)
	1157-1034 cm^{-1} (C-O *stretch*)
^{1}H NMR ($CDCl_3$)	δ 1,31 (3H, t, CH_3, *J*= 7 Hz), 1,46-1,85 (4H, m, CH_2), 2,11-2,25 (4H, m, CH_2), 2,31-2.33 (1H, m, CH), 4,10-4.22 (2H, q, CH_2, *J*= 7 Hz).
^{13}C NMR ($CDCl_3$)	δ 13,82 (CH_3), 25,71 (CH_2), 31,78 (CH_2), 36,59 (CH), 40,90 (CH_2), 41,99 (CH_2), 61,41 (CH_2), 170,99 (OC=O), 210,59 δ (C=O).

Synthesis of ethyl 2-(1,4-dioxaspiro[4,5]decan-7-yl)ethanoate

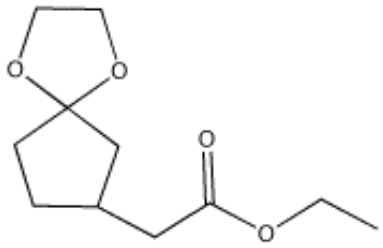


$C_{12}H_{20}O_4$ Mw 228,289

A mixture containing 33,6 mmol of ethyl-2-(3-oxyciclohexyl)ethanoate or ethyl-2-(3-oxyciclopentyl)ethanoate, 38,64 mmol of ethylene glycol, and 3,36 mmoli of p-TsOH monohydrate in 12 ml of toluene was heated at reflux for 16 h with continuous azeotropic removal of water and excess ethylene glycol by a Dean-Stark apparatus. The cooled mixture was washed with saturated aqueous sodium bicarbonate and saturated brine and was dried over anhydrous Na_2SO_4. The solution was concentrated in vacuum obtaining brown oils.

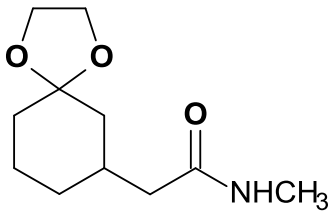
$C_{12}H_{20}O_4$	PM 228,289
Yield	88%
TLC	CH_2Cl_2 (10) Rf 0,37
IR neat	3515 cm^{-1} (C=O *stretch overtone*, br) 2937 cm^{-1} (C-H *stretch*) 1731 cm^{-1} (C=O *stretch*) 1174-1094-1071-1032 cm^{-1} (C-O *stretch*)
^{1}H NMR ($CDCl_3$)	δ 1,24 (3H, t, CH_3, J= 6,2 Hz), 1,39-1,75 (8H, m, CH_2), 2,12-2,18 (1H, m, CH), 2,27 (2H, s, CH_2), 3,95-3,94 (4H, s, CH_2, br), 4,15 (2H, q, CH_2, J= 6,6 Hz).
^{13}C NMR ($CDCl_3$)	δ 14,09 (CH_3), 22,68 (CH_2), 25,99 (CH_2), 30,99 (CH_2), 32,46 (CH), 40,89 (CH_2), 41,26 (CH_2), 59,96 (CH_2), 64,00 (CH_2-CH_2), 108,64 (C), 172,35 (OC=O).

Synthesis of ethyl 2-(1,4-dioxaspiro[4,4]nonan-7-yl)ethanoate

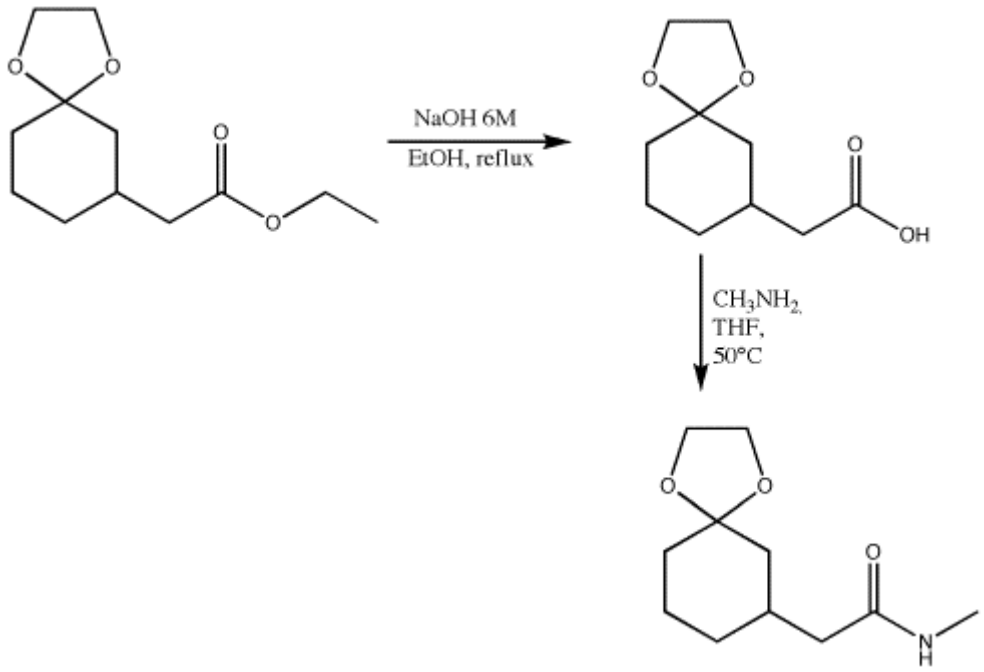


$C_{11}H_{18}O_4$	PM 214,258
Yield	80%
TLC	CH_2Cl_2 (10)
	Rf 0,42
IR neat	3535 cm^{-1} (C=O *stretch overtone*, br)
	2917 cm^{-1} (C-H *stretch*)
	1726 cm^{-1} (C=O *stretch*)
	1174-1032 cm^{-1} (C-O *stretch*)
^1H NMR ($CDCl_3$)	δ 1,43 (3H, t, CH_3, J= 6,7 Hz), 1,58-1,95 (8H, m, CH_2), 2,22-2,31 (1H, m, CH), 3,89-3,94 (4H, s, CH_2, br), 4,15 (2H, q, CH_2, J= 7 Hz).
^{13}C NMR ($CDCl_3$)	δ 14,19 (CH_3), 22,77 (CH_2), 25,39 (CH), 31,20 (CH_2), 40,92 (CH_2), 41,58 (CH_2), 59,97 (CH_2), 64,00 (CH_2-CH_2), 107,40 (C), 175,46 (OC=O).

Synthesis of 2-(1,4-dioxaspiro[4,5]decan-7-yl)-N-methylethanamide



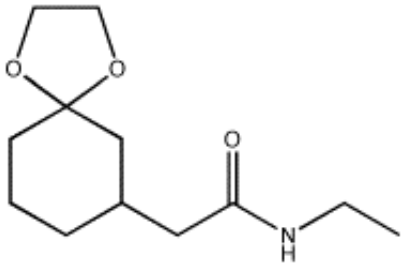
$C_{11}H_{19}NO_3$ Mw 213,274



The mixture of the corresponding ester, KOH/H_2O, and EtOH was refluxed for 2 h. After addition of 6N H_2SO_4 with ice-cooling the solution was saturated with NaCl and extracted with 3x100 ml of diethyl ether. The combined organic extracts were washed with 100 ml of brine and dried over Na_2SO4. After removing the solvent *in vacuo*, the remaining brown oils were used for the next step without further purification. A mixture of the acid and CDI in anhydrous THF was stirred until generation of CO_2 was completed. Then, the appropriate amine was added and the solution was refluxed for 8 h. After evaporating the solvent *in vacuo* the residue was dissolved in 200 ml of CH_2Cl_2 and the solution was consecutively washed with 3x100 ml of H_2O, 100 ml of 1N HCl and 2x250 ml of saturated Na_2CO_3 solution. After drying with Na_2SO_4 the solvent was removed *in vacuo*.

$C_{11}H_{19}NO_3$	PM 213,274
Yield	53%
TLC	CH_2Cl_2:MeOH 10:0,8
	Rf 0,45
IR (CH_2Cl_2)	3260-3089 cm^{-1} (NH *stretch*)
	2943.5 cm^{-1} (C-H *stretch*)
	1,657 cm^{-1} (C=O *stretch*)
^1H NMR ($CDCl_3$)	δ 1,16-2,32 (11H, m, $5CH_2$, 1CH), 2.78-2,81 (3H, d, CH_3), 3,93 (4H, s, CH_2, br), 5,65 (1H, s, NH, br).
^{13}C NMR ($CDCl_3$)	23,03 (CH_2), 26,27 (CH), 29,69 (CH_2), 33,14 (CH_3), 34,69 (CH_2), 41,08 (CH_2), 43,87 (CH_2), 63,68-64,22 (CH_2-CH_2), 108,89 (C), 172,47 (OC=O).

Synthesis of 2-(1,4-dioxaspiro[4,5]decan-7-yl)-N-ethylethanamide

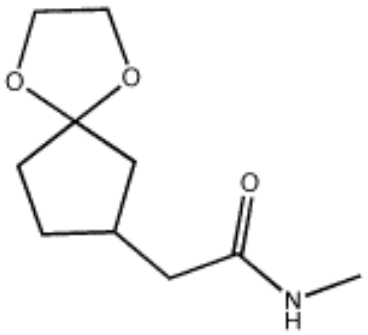


Mw 227,300 $C_{12}H_{21}NO_3$

$C_{12}H_{21}NO_3$	PM 227,300
Yield	50%
TLC	CH_2Cl_2:MeOH 10:0,8
	Rf 0,39
IR (CH_2Cl_2)	3282-3097 cm−1 (NH stretch)
	2973.5 cm^{-1} (C-H *stretch*)
	1,650 cm^{-1} (C=O *stretch*)
^1H NMR ($CDCl_3$)	δ 1,13 (3H, t, CH_3), 1,29-2,48 (11H, m, $5CH_2$, 1CH), 3,22-3,35 (4H, m, CH_2, br), 3,93 (2H, d, CH_2), 5,61 (1H, s, NH, br).
^{13}C NMR ($CDCl_3$)	δ 18,20 (CH), 22,68 (CH_2), 25,68 (CH_2), 31,41 (CH_3), 33,52 (CH_2), 34,68 (CH_2), 41,00 (CH_2),

43,91 (CH_2), 64,15-64,20 (CH_2-CH_2), 108,83 (C), 172,47 (OC=O).

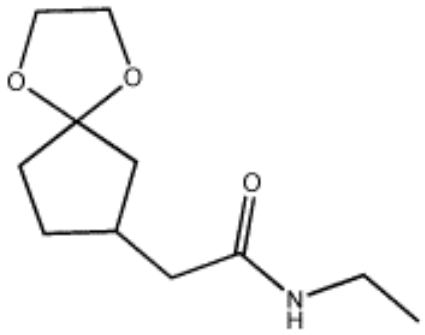
Synthesis of 2-(1,4-dioxaspiro[4,4]nonan-7-yl)-N-methylethanamide



Mw 199,247 $C_{10}H_{17}NO_3$

$C_{10}H_{17}NO_3$	PM 199,247
Yield	48%
TLC	CH_2Cl_2:MeOH 10:0,8 Rf 0,33
IR (CH_2Cl_2)	3272-3067 cm−1 (NH stretch) 29333.5 cm^{-1} (C-H *stretch*) 1,655 cm^{-1} (C=O *stretch*)
^{1}H NMR ($CDCl_3$)	δ 1,16-2,32 (9H, m, 4CH_2, 1CH), 2.78-2,81 (3H, d, CH_3), 3,93 (4H, s, CH_2, br), 5,66 (1H, s, NH, br).
^{13}C NMR ($CDCl_3$)	δ 23,03 (CH_2), 26,27 (CH), 29,69 (CH_2), 33,14 (CH_3), 34,69 (CH_2), 41,08 (CH_2), 43,87 (CH_2), 63,68-64,22 (CH_2-CH_2), 108,90 (C), 172,40 (OC=O).

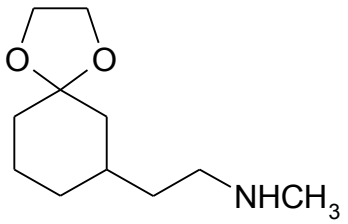
Synthesis of 2-(1,4-dioxaspiro[4,4]nonan-7-yl)-N-ethylethanamide



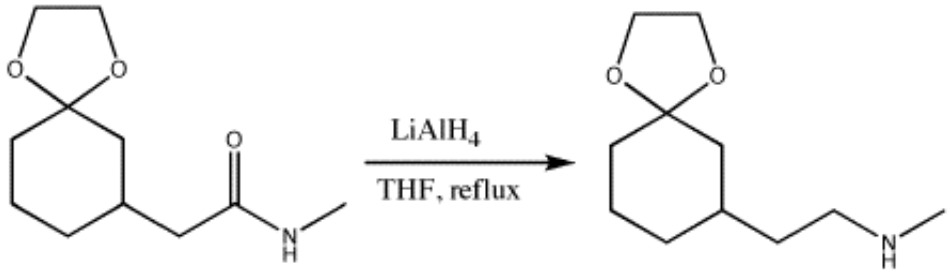
Mw 213,273 $C_{11}H_{19}NO_3$

$C_{11}H_{19}NO_3$	PM 213,273
Yield	46%
TLC	CH_2Cl_2:MeOH 10:0,8 Rf 0,39
IR (CH_2Cl_2)	3272-3067 cm−1 (NH stretch) 2943.5 cm^{-1} (C-H *stretch*) 1,652 cm^{-1} (C=O *stretch*)
^{1}H NMR ($CDCl_3$)	δ 1,16-2,32 (11H, m, $5CH_2$, 1CH), 2.78-2,81 (3H, d, CH_3), 3,93 (4H, s, CH_2, br), 5,66 (1H, s, NH, br).
^{13}C NMR ($CDCl_3$)	δ 21,00 (CH_2), 27,27 (CH), 29,69 (CH_2), 33,14 (CH_3), 38,69 (CH_2), 45,87 (CH_2), 51,08 (CH_2), 64,68-66,22 (CH_2-CH_2), 107,90 (C), 174,40 (OC=O).

Synthesis of 2-(1,4-dioxaspiro[4,5]decan-7-yl)-N-methylethanamine



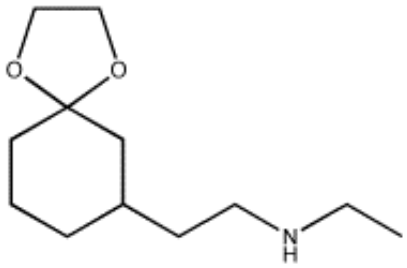
$C_{11}H_{21}NO_2$ Mw 199.290



To a solution of the amide in anhydrous THF was added $LiAlH_4$ during 20 min under stirring, ice cooling, and N_2. The mixture was allowed to warm up to ambient temperature, heated under reflux for 16 h, diluted with 100 ml of diethyl ether, and then cautiously poured into a mixture of 2N NaOH and diethyl ether under stirring, cooling, and N_2. After separating the organic layer, the aqueous layer was extracted with 3x150 ml of diethyl ether. The combined ether extracts were washed with 150 ml of saturated Na_2CO_3 solution, dried over Na_2SO_4, and evaporated *in vacuo*.

$C_{11}H_{21}NO_2$	PM 199,290
Yield	90%
TLC	CH_2Cl_2/MeOH/NH_3 10:0,3:0,1 Rf 0.41
IR (*neat*)	3368 cm^{-1} (N-H stretch) 2930 cm^{-1} (C-H stretch)
^{1}H NMR ($CDCl_3$)	δ 1,17-2,27 (11H, m, 5CH_2, 1CH), 2,59-2.62 (3H, d, CH_3), 3.64-3.75 (4H, m, CH_2), 3,94 (2H, s, CH_2, br), 5,15 (1H, s, NH, br).
^{13}C NMR ($CDCl_3$)	δ 23,09 (CH_2), 30,27 (CH), 31,77 (CH_2), 32,21 (CH_3), 34,73 (CH_2), 39,75 (CH_2), 41,67 (CH_2), 64.10-64,25 (CH_2-CH_2), 109,15 (C), 172,48 (OC=O).

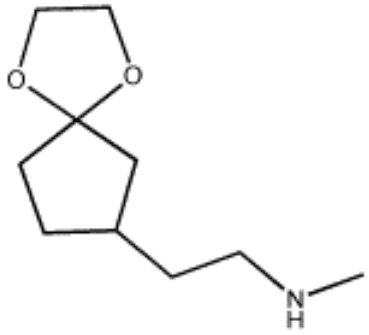
Synthesis of 2-(1,4-dioxaspiro[4,5]decan-7-yl)-N-ethylethanamine



$C_{12}H_{23}NO_2$ Mw 213,317

$C_{12}H_{23}NO_2$	PM 213,317
Yield	94%
TLC	CH_2Cl_2/MeOH/NH_3 10:0,3:0,1
IR (*neat*)	3368 cm^{-1} (N-H stretch)
^{1}H NMR ($CDCl_3$)	δ 1,11 (3H, t, CH_3), 1,19-1,74 (11H, m, 5CH_2, 1CH), 2,66 (4H, q, CH_2), 3.940 (4H, s, CH_2), 5,15 δ (1H, s, NH, br).
^{13}C NMR ($CDCl_3$)	δ 15,23 (CH), 23,15 (CH_2), 31,84 (CH_2), 33,70 (CH_3), 34,75 (CH_2), 41,79 (CH_2), 44,15 (CH_2), 47,34 (CH_2), 49,34 (CH_2), 64,09-64,26 (CH_2-CH_2), 109,23 (C),

Synthesis of 2-(1,4-dioxaspiro[4,4]nonan-7-yl)-N-methylethanamine

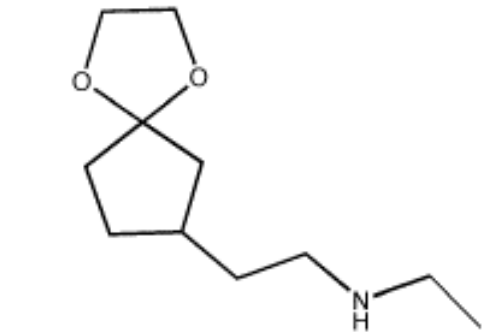


$C_{10}H_{19}NO_2$ Mw185,263

$C_{10}H_{19}NO_2$	PM 185,263
Yield	90%

TLC	CH_2Cl_2/MeOH/NH_3 10:0,3:0,1 Rf 0.41
IR (*neat*)	3368 cm^{-1} (N-H *stretch*) 2930 cm^{-1} (C-H *stretch*)
^1H NMR ($CDCl_3$)	δ 1,3-2,57 (9H, m, 4CH_2, 1CH), 2,69-2.82 (3H, d, CH_3), 3.54-3.70 (4H, m, CH_2), 3,84 (2H, s, CH_2, br), 5,05 (1H, s, NH, br).
^{13}C NMR ($CDCl_3$)	δ 22,06 (CH_2), 30,07 (CH), 31,07 (CH_2), 32,81 (CH_3), 34,03 (CH_2), 40,67 (CH_2), 63.90-64,15 (CH_2-CH_2), 108,15 (C), 174,48 (OC=O).

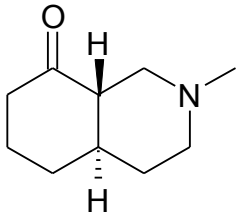
Synthesis of 2-(1,4-dioxaspiro[4,4]nonan-7-yl)-N-ethylethanamine



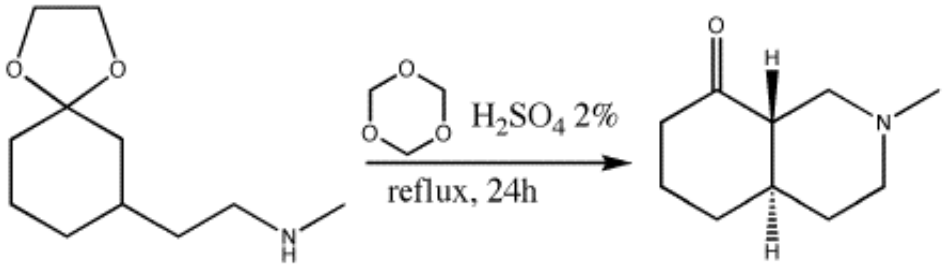
$C_{11}H_{21}NO_2$ Mw 199,157

$C_{11}H_{21}NO_2$	PM 199,157
Yield	92%
TLC	CH_2Cl_2/MeOH/NH_3 10:0,3:0,1 Rf 0.43
IR (*neat*)	3368 cm^{-1} (N-H *stretch*) 2930 cm^{-1} (C-H *stretch*)
^1H NMR ($CDCl_3$)	δ 1,01 (3H, t, CH_3), 1,39-1,84 (9H, m, 4CH_2, 1CH), 2,56 (4H, q, CH_2), 3.95 (4H, s, CH_2), 5,19 δ (1H, s, NH, br).
^{13}C NMR ($CDCl_3$)	δ 14,93 (CH), 22,17 (CH_2), 30,98 (CH_2), 33,70 (CH_3), 34,75 (CH_2), 41,79 (CH_2), 43,15 (CH_2), 45,34 (CH_2), 64,00-64,36 (CH_2-CH_2), 108,23 (C), 175,50 (OC=O).

Synthesis of (4□RS,8□SR)-2-methyloctahydroisoquilin-8(8aH)-one



$C_{10}H_{17}NO$ Mw 167,248

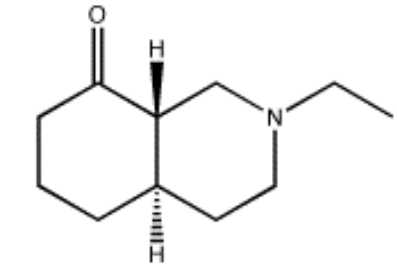


To a solution of mmol 1,3,5-trioxane in H_2SO_4 2% refluxed (105 °C), was added dropwise during 3 h mmol of amine. The reflux is maintained for 24 h. After cooling, the solution is washed with CH_2Cl_2 (2x30 ml), basified with NaOH 40% and extracted with other CH_2Cl_2 (2x30 ml). The combined organic layers are dried over Na_2SO_4 and concentrated *in vacuo*. The resulting product was undertaken to chromatographic purification using as eluents chloroform and methanol (10:0,5) obtaining yellow oils.

$C_{10}H_{17}NO$	PM 167.248
Yield	66%
TLC	CH_3Cl/MeOH 10:0,5 Rf 0,3
IR ($CHCl_3$)	2932 cm−1 (C-H *stretch*) 17064 cm−1 (C=O *stretch*)
^{1}H NMR ($CDCl_3$)	1,22-1,99 δ (8H, m, $4CH_2$), 2,10-2,30 (1H, m, CH_2), 2,31 (3H, s, CH_3), 2,17 -2,39 δ (3H, m, $2CH_2$), 2,83 (1H, m), 3,05 (1H, m).
^{13}C NMR ($CDCl_3$)	δ 26,24 (CH_2), 31,64 (CH_2), 32,86 (CH_2), 34,35

	(CH_3), 42,66 (CH), 41,36 (CH_2), 54,67 (CH_2), 55,31 (CH_2-CH_2), 108,83 (C), 211,10 (OC=O).
MS (MeOH)	*m/z* 168,3

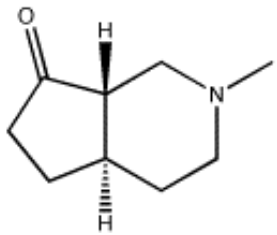
Synthesis of (4□RS,8□SR)-2-ethyloctahydroisoquilin-8(8aH)-one



$C_{11}H_{19}NO$ Mw181,275

$C_{11}H_{19}NO$	PM 181.275
Yield	50%
TLC	$CHCl_3$:MeOH 10:0,5 Rf 0,44
^{1}H NMR ($CDCl_3$)	δ 1,12 (3H, t, CH_3, *J*=6,8 Hz), 1,22-1,98 δ (8H, m, 4 CH_2), 2,07-2,32 (1H, m, CH_2), 2,32 (2H, q, CH_2, *J*=6,8 Hz), 2,17 -2,39 (3H, m, 2 CH_2), 2,83 (1H, m), 3,05 (1H, m).
^{13}C NMR ($CDCl_3$)	δ 12,02 (CH), 26,36 (CH_2), 31,85 (CH_2), 32,98 (CH_2), 41,50 (CH_2), 43,79 (CH_2), 43,46 (CH_3), 52,61 (CH_2), 53,10 (CH_2), 53,46 (CH_2), 211,38 (OC=O).
MS (MeOH)	*m/z* 182,2

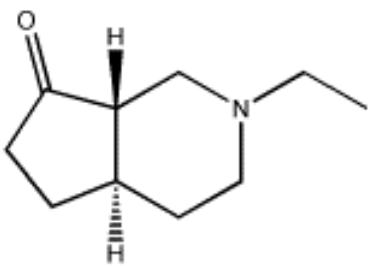
Synthesis of (4□RS,7□SR)-2-methylhexa-1H-cyclopental[c]pyridine-7(7aH)-one



$C_9H_{15}NO$ Mw 153,222

$C_9H_{15}NO$	PM 153.222
Yield	66%
TLC	CH_3Cl/MeOH 10:0,5 Rf 0,39
IR ($CHCl_3$)	2932 cm−1 (C-H *stretch*) 17064 cm−1 (C=O *stretch*)
^1H NMR ($CDCl_3$)	δ 1,15-1,96 (6H, m, $3CH_2$), 2,07-2,30 (1H, m, CH_2), 2,38 (3H, s, CH_3), 2,39-2,73 (3H, m, $2CH_2$), 2,83 (1H, m), 3,05 (1H, m).
^{13}C NMR ($CDCl_3$)	δ 26,24 (CH_2), 42,66 (CH), 31,64 (CH_2), 32,86 (CH_2), 41,36 (CH_2), 54,67 (CH_2), 55,31 (CH_2-CH_2), 108,83 (C), 211,10 (OC=O).

Synthesis of (4□RS,7□SR)-2-ethylhexa-1H-cyclopental[c]pyridine-7(7aH)-one

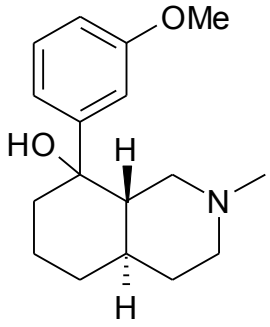


$C_{10}H_{17}NO$ Mw 167,248

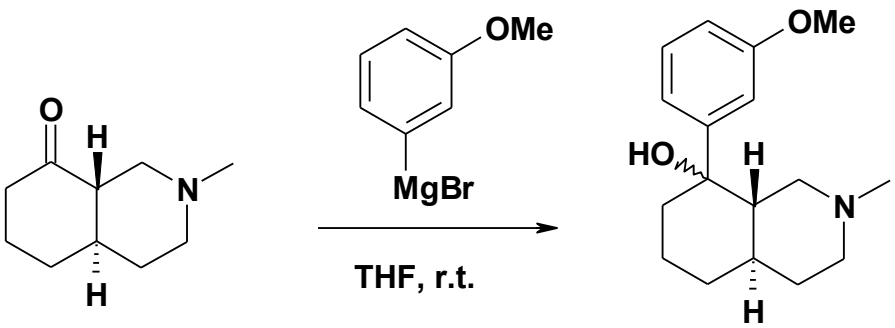
$C_{10}H_{17}NO$	PM 167.248
Yield	66%

TLC	$CH_3Cl/MeOH$/ 10:0,5 Rf 0,3
IR ($CHCl_3$)	2932 cm−1 (C-H stretch) 17064 cm−1 (C=O chetonico stretch)
^1H NMR ($CDCl_3$)	δ 1,32 (3H, t, CH_3, *J*=6,8 Hz), 1,42-1,98 δ (6H, m, 4 CH_2), 2,17-2,32 (1H, m, CH_2), 2,30 (2H, q, CH_2, *J*=6,8 Hz), 2,17 -2,39 (3H, m, 2 CH_2), 2,83 (1H, m), 3,15 (1H, m).
^{13}C NMR ($CDCl_3$)	δ 12,62 (CH), 26,96 (CH_2), 30,85 (CH_2), 31,98 (CH_2), 40,50 (CH_2), 42,46 (CH_3), 51,61 (CH_2), 52,10 (CH_2), 53,46 (CH_2), 210,38 (OC=O).

Synthesis of (4αR/S,8αS/R)-8R/S-(3-methoxyphenyl)-2-methyldecahydroisoquinolin-8-ol



$C_{17}H_{25}NO_2$ Mw 275.386

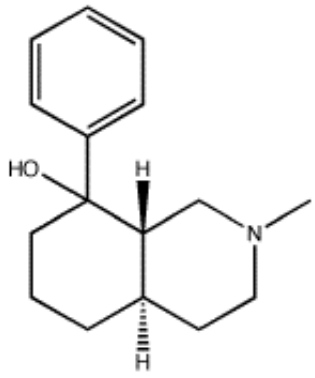


At the temperature of 0°C/-10°C, to a solution of 3-methoxyphenylmagnesium bromide 1M in THF or phenylmagnesium

bromide 1M in THF was added under vigorous stirring a solution of *trans*-decahydroisoquinoline and octahydro-1*H*-cyclopenta[c]pyridine derivatives in THF. The mixture was stirred for 4 hours at rt and then poured into a mixture formed of 0.478 g di NH_4Cl, 0.95 ml of H_2O and 1g of ice. The resulting two layers were separated and the aqueous layer was extracted two times with diethyl ether. Combined organic layers were dried over Na_2SO_4, and concentrated *in vacuo*, obtaining colourless o weakly yellow oils. Crude products were undertaken to flash cromatography using as eluents chloroform and methanol 10:0,8.

$C_{17}H_{25}NO_2$	PM 275,386
Yield	40%
TLC	$CHCl_3$:EtOH 10:1 Rf 0,45
^{1}H NMR ($CDCl_3$)	δ 1,24-2,52 (14H, m, 6CH_2, 2CH), 2,74 (3H, s, CH_3), 2,27 (3H, s, CH_3), 5,51 δ (1H, br, OH), 6,75-7,38 (4H, m, CH).

Synthesis of (4αR/S,8αS/R)-2-methyl-8R/S-phenyldecahydroisoquinolin-8-ol

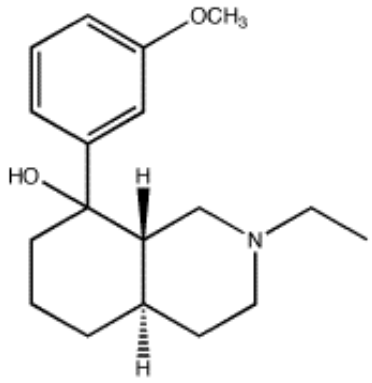


$C_{16}H_{23}NO$ Mw 245,360

$C_{16}H_{23}NO$	PM 245,360

Yield	45%
TLC	$CHCl_3$:EtOH 10:1 Rf 0,35
^1H NMR ($CDCl_3$)	δ 1,24-2,52 (14H, m, $6CH_2$, 2CH), 2,27 (3H, s, CH_3), 5,52 (1H, br, OH), 6,75-7,38 (5H, m, CH).

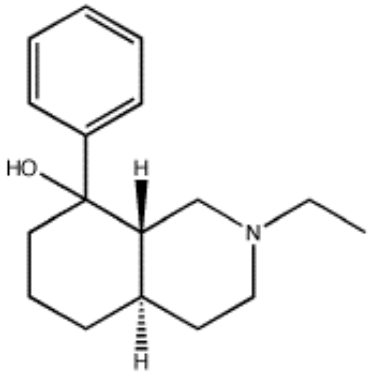
Synthesis of (4αR/S,8αS/R)-2-ethyl-8R/S-(3-methoxyphenyl)-decahydroisoquinolin-8-ol



$C_{18}H_{27}NO_2$ Mw 289,412

$C_{18}H_{27}NO_2$	PM 289,412
Yield	40%
TLC	$CHCl_3$:MeOH 10:0,5 Rf 0,33
^1H NMR ($CDCl_3$)	δ 0,97 (3H, t, CH_3, *J*=7,2 Hz), 1,19-2,22 (14H, m, $6CH_2$, 2CH), 2,49 (2H, q, CH_2, *J*=7,2 Hz), 2,73 (3H, s, CH_3), 5,52 (1H, br, OH), 6,735-7,564 (4H, m, CH).

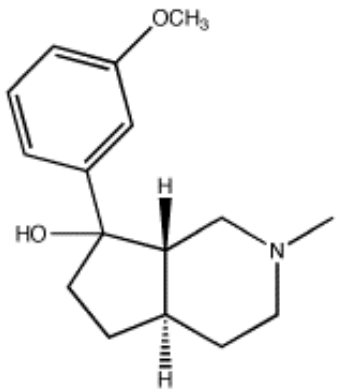
Synthesis of (4αR/S,8αS/R)-2-ethyl-8R/S-phenyldecahydroisoquinolin-8-ol



$C_{17}H_{25}NO$ Mw 259,386

$C_{17}H_{25}NO$	PM 259,386
Yield	40%
TLC	$CHCl_3$:MeOH 10:0,5 Rf 0,33
^1H NMR ($CDCl_3$)	δ 0,98 (3H, t, CH_3, *J*=7,2 Hz), 1,21-2,22 (14H, m, $6CH_2$, 2CH), 2,54 (2H, q, CH_2, *J*=7,2 Hz), 5,42 δ (1H, br, OH), 6,735-7,564 (5H, m, CH

Synthesis of (4aRS,7aSR)-7-(3-methoxyphenyl)-2-methyloctahydro-1H-cyclopenta[c]pyridin-7-ol

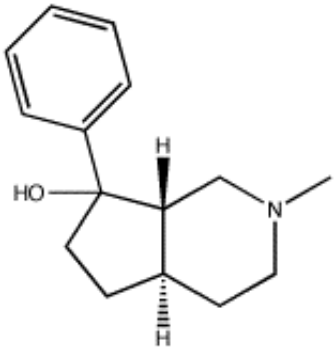


$C_{16}H_{23}NO_2$ Mw 261,359

$C_{16}H_{23}NO_2$	PM 261,359

Yield	41%
TLC	$CHCl_3$:MeOH 10:0,5 Rf 0,36
^1H NMR ($CDCl_3$)	δ 1,21-2,42 (12H, m, $6CH_2$, 2CH), 2,64 (3H, s, CH_3), 2,37 (3H, s, CH_3), 5,41 δ (1H, br, OH), 6,65-7,47 (4H, m, CH).

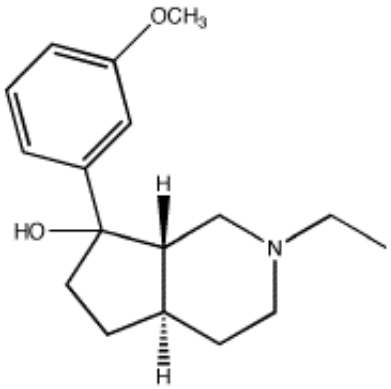
Synthesis of (4aR/S,7aS/R)-2-methyl-7R/S-phenyloctahydro-1H-cyclopenta[c]pyridin-7-ol



$C_{15}H_{21}NO$ Mw 231,333

$C_{15}H_{21}NO$	PM 231,333
Yield	39%
TLC	$CHCl_3$:MeOH 10:0,5 Rf 0,33
^1H NMR ($CDCl_3$)	δ 0,98-2,29 (12H, m, $6CH_2$, 2CH), 2,34 (3H, s, CH_3), 5,32 (1H, br, OH), 6,59-7,48 (5H, m, CH).

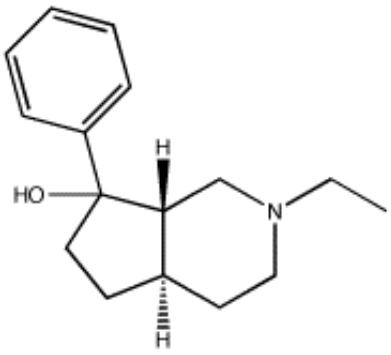
Synthesis of (4aR/S,7aS/R)-2-ethyl-7R/S-(3-methoxyphenyl)-octahydro-1H-cyclopenta[c]pyridin-7-ol



$C_{17}H_{25}NO_2$ Mw 275,386

$C_{17}H_{25}NO$	PM 275,386
Yield	44%
TLC	$CHCl_3$:MeOH 10:0,5 Rf 0,38
^1H NMR ($CDCl_3$)	δ 1,08 (3H, t, CH_3, *J*=7,2 Hz), 1,19-2,22 (12H, m, $6CH_2$, 2CH), 2,59 (2H, q, CH_2, *J*=7,2 Hz), 2,83 (3H, s, CH_3), 5,22 (1H, br, OH), 6,73-7,56 (4H, m, CH).

Synthesis of (4aR/S,7aS/R)-2-methyl-7R/S-phenyloctahydro-1H-cyclopenta[c]pyridin-7-ol



$C_{16}H_{23}NO$ Mw 245,360

$C_{16}H_{23}NO$	PM 245,360
Yield	45%
TLC	$CHCl_3$:MeOH 10:0,5 Rf 0,49
^1H NMR ($CDCl_3$)	δ 1,17 (3H, t, CH_3, *J*=7,2 Hz), 1,09-2,13 (12H, m, $6CH_2$, 2CH), 2,63 (2H, q, CH_2, *J*=7,2 Hz), 5,69 (1H, br, OH), 6,72-7,59 (5H, m, CH).

*

[*] **ABBREVIATIONS**: MOR, μ-opioid receptor; DOR, δ-opioid receptor; KOR, κ-opioid receptor; Tramadol HCl, (1*RS*,2*RS*)-2-[(dimethyl-amino)methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride; 5-HT, serotonin; NE, norepinephrine; NSAIDs, non-steroidal antinflammatories; i.v., intravenous; CNS, central nervous system; i.m., intramuscular; i.a., intrarticular; s.c., subcutaneous; CYP2D6 sparteine oxygenase; ED_{50}, effective dose; SPR, Substance P receptor; PKC, protein kinase C; $LiAlH_4$, litiul aluminium hydride; THF, tetrahydrofuran; NMR, nuclear magnetic resonance; IR, infrared spectroscopy; MS, mass spectroscopy; TLC, thin layer chromatography; TMS, tetramethylsilane; DMF, dimethylformamide; LiCl, litium chloride; AcOH, acetic acid; KOH, potassium hydroxide; EtOH, ethanol; H_2SO_4, sulfuric acid; CDI, N,N-carbonyldiimidazole; iPrMgCl, isopropyl magnesium chloride; rt, room temperature; I_2, iodine; p-TsOH, p-toluensulfonic acid; NH_4Cl, ammonium chloride

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