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Design and synthesis of new vinca alkaloid derivatives as potential sigma-2 receptor ligands

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DOCTORATE THESIS

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Introduction

Sigma receptors were first proposed in the mid-1970s thanks to the studies of Martin and co-workers (1976). They demonstrated that the mania syndrome observed on animal models, after treatment with the bezomorphanic derivative (±)-*N*-allyl-normetazocine (code number: (±)-SKF 10,047) (Fig. 1), was due by interaction with an opioid receptor subclass until then unknown. In fact, they distinguished three opioid subclasses according to the interaction with three different agonists: morphine (μ receptor agonist), ketocyclazocine (κ receptor agonist) and (±)-SKF 10,047 (σ receptor agonist).[1]
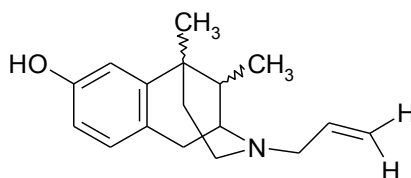

Figure 1: (±)-SKF 10,047

The hypothesis of a new opioid subclass was not confirmed when enantioselectivity studies were carried out on benzomorphan derivatives. Brady et al. demonstrated that sigma receptor showed an enantiomeric selectivity for (+)-isoforms of benzomorphans[2] while μ and κ opioid receptors preferred the (−)-isoforms.[3] Additionally, opioid antagonists such as naloxone e naltrexone were not able, both *in vivo* and *in vitro,* to inhibit the sigma ligands effects. [4,5] These pharmacological evidences produced the idea that the psychotomimetic effects of SKF-10047 were mediated not by an opioid-sigma receptor, as Martin proposed, but by a different receptor system.

The high affinity of (+) SKF-10,047 (Fig. 2) for the binding site of the NMDA antagonist 1-phenylcyclohexyl-piperidine (PCP) (Fig. 2), led to the co-identification of the sigma receptor with PCP binding site.[6] The Sigma-PCP hypothesis fell through when the

antipsychotic haloperidol (Fig. 2) demonstrated the inability to displace [3H] PCP from its binding site, even though it had high affinity for [3H] (+) SKF 10,047 labelled sites ($K_i = 4$ nM in rat brain membranes).[7] Hence it was concluded that [3H](+)-SKF10,047 bound at two different sites: the haloperidol-sensitive site and the PCP-sensitive site, respectively with high and low affinity.

PCP   **(+)-SKF-10,047**   **Haloperidol**
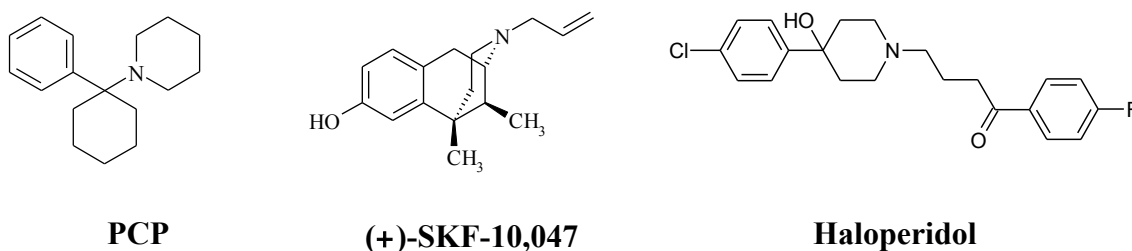
Figure 2

Even a co-relationship with dopaminergic sites was proposed because of the haloperidol and other antipsychotic drugs activity on sigma sites. [8]

The confusion about the identity of sigma sites was due to the lack of selective ligands in the pharmacological studies. When new high affinity and high selectivity ligands, such as 1,3-Di(2[5-3H] tolyl) guanidine (DTG) and (+)-[3H]-3-[3-hydroxyphenyl]-N-(1-propyl)piperidine (3-PPP) (Fig.3) were discovered, many doubts about the knowledge of sigma receptors were resolved. [9,10]

DTG   **3-PPP**

Figure 3

Sigma receptor subclasses

At the moment a number of studies confirm the existence of multiple binding sites named sigma-1 and sigma-2.[11,12] The two subclasses are mainly determined by their different selectivity toward some ligands.

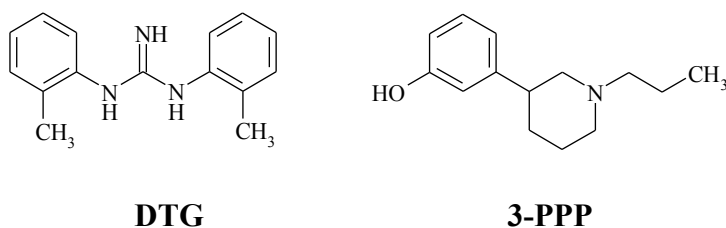Although initial evidences suggested a co-identity of (+)-3-PPP and DTG binding sites, further studies indicated a diverse drug-specificity of both sites. Indeed, binding studies on C57BL/6 mouse brain membranes showed that (+)-3-PPP and haloperidol possessed an affinity 20-30 fold higher for [3H] (+)-3-PPP sites than for [3H]-DTG sites. Instead, (+)-pentazocine and (+)-SKF10047 possessed a nano-molar affinity for [3H](+)-3-PPP sites, and respectively, micro-molar and null (with a concentration of 10 μM) for [3H]-DTG sites (table 1).[13] The same authors obtained similar results studying some of the inhibitors of mono amino oxidase (MAO). The selective MAO A inhibitor clorgiline showed an affinity for [3H](+)-3-PPP sites 159 fold higher than for [3H] DTG sites (table 2). Also Deprenyl, MAO B inhibitor, produced similar results, however its two stereoisomers differed for the affinity toward [3H](+)-3-PPP sites.[14] All together these data, supported the existence of two subclasses: **sigma-1** receptor corresponding to [3H](+)-3-PPP and [3H](+)-SKF-10047 binding sites and **sigma-2** receptor corresponding to [3H] DTG binding site.

Table 1. Comparison of affinity data of sigma ligands toward [³H](+)- 3-PPP e [³H]DTG sites in C57BL/6 mouse brain membranes. K_i and nH are respectively the affinity constant and Hill's coefficient.

Ligands	[³H](+)-3- PPP		[³H]DTG		
	K_i (nM)	nH	K_i (nM)	nH	K_i ratio
Haloperidol	2.2 ± 0.2	1.02	73 ± 7	0.95	33
(+)-Pentazocine	5.7 ± 0.3	0.89	3689 ± 450	0.71	647
(+)-3-PPP	9.5 ± 1.0	0.83	225 ± 20	0.78	22
(+)-SKF-10047	15 ± 2.0	0.84	>10 000	−	>600
DTG	53 ± 4.0	0.93	32 ± 3	0.88	0.6
(−)-Pentazocine	73 ± 6.0	0.87	380 ± 23	0.68	5.2
(−)-3-PPP	310 ± 25	0.84	1200 ± 85	0.70	4
(−)-SKF-10047	510 ± 47	0.90	3250 ± 200	0.88	6

Table 2. Comparison of affinity constants of MAO inhibitors for [³H](+)-3-PPP, [³H](+) SKF-10047 and [³H]DTG binding sites in C57BL/6 mouse brain membranes.

Ligands	[³H](+)-3-PPP	[³H](+)-SKF10047	[³H]DTG
	K_i (nM)	K_i (nM)	K_i (nM)
Clorgiline	3.2 ± 0.3	2.9 ± 0.3	505 ± 43
(+)-Deprenyl	79 ± 6	82 ± 7	1880 ± 120
(−)-Deprenyl	287 ± 22	310 ± 30	2100 ± 130
Harmaline	510 ± 45	470 ± 50	4100 ± 220
Ro11-1163	980 ± 78	860 ± 85	>50 000
Ro11-1049	1450 ± 92	1800 ± 150	>50 000
Pargiline	3200 ± 150	3750 ± 280	6700 ± 700
Tranilcipromine	6150 ± 480	6700 ± 680	8300 ± 680

The stereo-selectivity is one of the most important issues regarding the interaction ligand-receptor. As shown before, the dextrorotatory isomers of benzomorphan including (+)-pentazocine, (+)-cyclazocine, (+)-SKF-10,047 and their related, possess a higher affinity on sigma sites than levorotatory isomers, which are more selective for opioid sites. Though considering the sigma subclasses, this is valid only for sigma-1 receptor and not for sigma-2 receptor, which tends to have a slight preference for (−)-stereoisomers of benzomorphans (table 3).

Bowen and co. demonstrated, through binding assays in homogenate rat brain, that the ligand-specificity of [³H](+)-pentazocine sites, agreed with the already known sigma-1 properties. While the ligand-specificity of [³H] DTG sites, in the presence of

dextrallorphan, sigma-1 binding blocker, agreed with sigma-2 properties (table 3).[15]

Moreover the specificity profile of [^{3}H](+)-pentazocine sites in rat brain was identical to

[^{3}H]-3-PPP sites in C57BL/6 mouse brain (table 2).

Table 3. Constants of affinity of sigma ligands for sigma-1 and sigma-2 sites in rat brain.[a]

Ligands	[^{3}H](+)-Pentazocine	[^{3}H]DTG[b]	
	K_i (nM)	K_i (nM)	K_i ratio
Haloperidol	1.9 ± 0.3	79.8 ± 20	42
(+)-Pentazocine	6.7 ± 1.2	1361 ± 134	203
(+)-SKF-10047	28 ± 2.8	33654 ± 9409	1201
(−)-Pentazocine	44 ± 1.2	108 ± 6	2.4
(+)-Cyclazocine	66 ± 10	7718 ± 864	117
DTG	74 ± 15	61 ± 13	0.8
(+)-3-PPP	79 ± 3	120 ± 24	1.5
(−)-Cyclazocine	472 ± 28	656 ± 16	1.4
(−)-SKF-10047	779 ± 128	2283 ± 812	2.9

[a] Adapted by Bowen and co-workers (1993). [b] The [^{3}H]DTG binding was carried out in presence of dextrallorphan (1 μM).

The pharmacological classification of sigma receptor types was also determined by

differences in histological and subcellular distribution.[16,17]

Anatomical distribution and related functions

The knowledge of sigma receptors location is very helpful in the identification and the understanding of their physiological functions. In order to individuate sigma receptors in different body areas several studies were carried out through autoradiography with labelled ligands, such as [^{3}H]-DTG, [^{3}H]-NE-100, [^{3}H]-(+)-SK&F 10,047, [^{3}H]-(+)-3-PPP, and through PET (positrons emission tomography).

Nervous System

The highest concentration of sigma receptors was found in the motor nuclei of the brain stem. High levels were also discovered in the cranial, facial, hypoglossal and trigeminal nerves. Alike the cerebellum, red nucleus and grey matter of spinal cord, were found particularly rich in sigma receptors, demonstrating their involvement in motor function.[18,19,20]

Confirming their involvement in the modulation of motor behaviour, moderate levels of sigma receptors were found in substantia nigra neurons through nigro-striatal dopaminergic mechanisms.[21,22]

The presence of sigma-1 receptors in the dentate gyrus and in the stratum pyramidale cells supports their involvement in the learning and memory processes.[23]

Sigma receptors are located in the olfactory bulb, in limbic and paralimbic areas, such as prefrontal cortex, hypothalamic region and amygdala. These distinct locations suggest an important role of sigma receptors in the modulation of affective states.[24]

Likewise the neuroendocrine areas are rich in sigma receptors, particularly the supraoptic and paraventricular nuclei, implying a role in the control of the hormonal secretion. [25]

Several regions of the visual system have a significant presence of sigma receptor, such as the superior colliculus and lateral geniculate, proving an important role in visual function.[24]

Sigma receptors are present both in the ganglions of the dorsal radix of the spinal chord and in the central grey of the midbrain, assisting in the modulation of pain perception.[20,25]

Information about the distribution in cerebral areas derived also from the binding studies illustrated in table 4.[26,27]

Table 4. Sigma-1 and sigma-2 binding sites in different cerebral areas (fmol/mg protein)

Cerebral Areas	$\sigma_1{}^a$	$\sigma_2{}^b$	σ_1/σ_2
Hypothalamo	90,7 ± 10,2	163,0 ± 9,5	1,86 ± 0,17
Thalamo	71,3 ± 11,1	157,8 ± 23,8	2,22 ± 0,04
Septum	43,9 ± 3,4	113,7 ± 10,0	2,60 ± 0,10
Hippocampus	63,0 ± 7,2	164,7 ± 18,6	2,64 ± 0,12
Corpus Striatum	65,5 ± 10,9	167,4 ± 15,7	2,68 ± 0,19
Cortex	89,7 ± 16,8	280,6 ± 42,8	3,23 ± 0,16
Cerebellum	74,4 ± 7,8	261,0 ± 32,4	3,51 ± 0,29
Midbrain	110,9 ± 12,7	188,3 ± 18,7	1,72 ± 0,10
Telencephalon	202,8 ± 21,9	304,5 ± 34,5	1,63 ± 0,18
Guinea pig brain	80,6 ± 18,2	54.5 ± 22.5	0,67 ± 0,08

[a] Sigma-1 sites labelled with [^{3}H]-(+)-Pentazocine. [b] Sigma-2 sites labelled with [^{3}H]-DTG in presence of dextrallorphan (1μM)

Peripheral organs

The heart contains high levels of sigma receptors, which 80% consist of sigma-1 type. In myocites sigma receptors influence contractility, calcium influx and cardiac rhythm. [28,29] In intracardiac neurons both subtypes regulate the excitability, modulating calcium and potassium channels.[30,31]

The highest concentration of sigma receptors in the body has been found in the liver, where both subtypes are present. However, the function of sigma receptors in the liver, as well as in the kidney, is yet unknown.[32]

Another part of the body that contains high levels of sigma-1 receptor is the spleen. Moreover the presence of sigma-1 receptor both in the spleen and immune cells would explain their role in immune system regulation.[33,34]

Recently, sigma-1 receptors were found in the ciliary body and retina of the eye, justifying the effects of sigma agonists in reducing ocular pressure and in retina protection against cell death. [35,36]

Significant levels of sigma receptors are found in highly proliferative cells such as blood and tumour cells. Blood cells expressing sigma receptors are: granulocytes, lymphocytes, and natural killer cells. [37] Tumour cells overexpress sigma receptors, especially in proliferating tumours. Indeed, sigma agonists have been shown to be useful as probes for diagnostic imaging and as anticancer agents because they are able to determine tumour cell death via apoptotic mechanism.[38,39,40]

## Proposed endogenous ligands for sigma receptors

In spite of the significant advances achieved in the pharmacological characterization and in the knowledge of sigma receptors action mechanisms, the sigma endogenous ligands are still unknown.

Neurosteroids are the main candidates for the role of endogenous ligands of sigma receptors. Su T.P. and co. (1988) reported the nanomolar affinity of progesterone for sigma sites, because they observed that the neurosteroid inhibited the binding of both $[^3H](+)$-SKF10.047 (Ki = 268 nM) and $[^3H]$-haloperidol, in cavia brain and in cavia spleen respectively.[41]

Subsequently, it was noted that the binding of $[^3H]$-progesterone, used as radiolabeled ligand in different tissues, was inhibited by other sigma receptor ligands such as haloperidol, carbopentane, DTG, (+)-3-PPP and rimcazole.[42,43] Different neurosteroids, possessing micromolar affinity for sigma receptors, were considered, but it is still not clarified if their physiological effects are mediated by interaction with sigma receptors.[41,42] The limited functional studies only explain that some of them act as sigma-1 agonist (e. g. pregnenolone, Fig. 4) while others as sigma-1 antagonist (e. g. progesterone, Fig.4).[44] Thus, the current evidences are far from identifying neurosteroids as endogenous ligands of sigma receptors. On the other hand a number of studies have identified sigma-1 receptor as a molecular target, at the structural, biochemical and physiological levels, for neuro(active)steroids actions.[45]

Other hypothesized endogenous ligand was the Neuropeptide Y that has been reported to have significant affinity for sigma receptors.[46] Both Neuropeptide Y and peptide YY showed the ability to displace $[^3H](+)$-SF&K10.047 from its binding sites with an IC_{50} around 10 nM. Unfortunately, subsequent studies did not confirm these results.[47]

A number of studies have shown that several divalent cations inhibit radioligands binding to sigma receptors. The cations considered include magnesium, calcium, zinc, cadmium and copper. Among these cations, some preferred the sigma-1 receptor while others the sigma-2 type.[48,49] Another noteworthy finding was acquired from a binding study, performed under physiological conditions, where depolarization caused the release of zinc, from hippocampal slices, displacing [³H] DTG but not [³H](+)-pentazocine. This evidence suggested that zinc could be an endogenous ligand for sigma-2 receptor.[49]
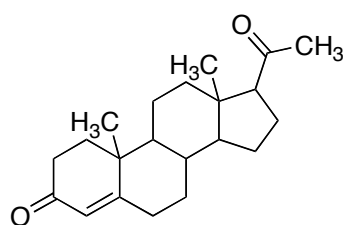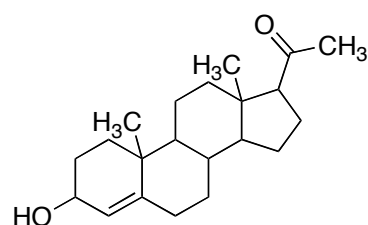
Progesterone **Pregnenolone**

Figure 4: neurosteroids acting on sigma-1 receptor.

Sigma-1 receptor

Characterization

So far, significant advances have been made in the characterization of sigma-1 receptors, not only with pharmacological methods but also in the biochemical field.

First steps were made through photoaffinity labelling with [^{3}H] azido-DTG that showed a specific binding capacity toward a 29 KDa polypeptide found in NCB-20 cells and guinea pig cerebral membranes.[50,51]

McCann and Su (1991) isolated a CHAPS* solubilized sigma site complex (450 KDa), from rat liver membranes, which possessed all the pharmacological features of a neuronal sigma 1 receptor.[52]

The sigma-1 receptor has been recently cloned from various sources, including guinea-pig liver,[53] human brain,[54] rat brain,[55] mouse brain[56] and human placental choriocarcinoma cells.[57] The gene expressing sigma-1 receptor is 7 Kbp long and has four exons and three introns and it is located in the human chromosome 9.[54]

The receptor possesses a sequence of 223 amino acidic residues and does not show homology neither with opioid receptors nor with other neurotransmitter receptors.

Sigma 1 receptor shares significant amino acid sequence similarities with the yeast sterol C8 ± C7 isomerase (ERG2 protein). Pharmacologically, but not structurally, the sigma-1 site is also related to the emopamil binding protein, the mammalian sterol C8 ± C7 isomerase.[58] This was confirmed by studies on HMM-HMM® and BLAST® (Basic Local Alignment Search Tool).[59,60] Recently, a significant sequence homology (35% and 45 %) was found also with another two enzymes involved in biosynthesis of steroids including

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* 3-[(3-cholamidopropyl) dimethylamino]-1-propanesulfonate

isopentenyl diphosphate isomerase and 17β-estradiol dehydrogenase.[61,62] On the contrary, sigma-1 receptors do not possess sterol isomerase activity[53] and the sequence homology could be explained by a conservative evolution hypothesis.

Aydar and co-workers (2004) proposed a model for sigma-1 receptor (Fig. 5) that consist of two transmembrane spanning regions, an extracellular loop with 50 amino acids, an intracellular C-terminal segment with 125 amino acids and a short intracellular N-terminal segment (10 amino acids). The C-terminal extremity (blue labelled in Fig. 5) has complete homology with the yeast sterol isomerase. The pair Arg-Arg (red labelled in the Fig. 5), typically found in endoplasmic reticulum (ER) receptors, is a retention signal that directs the retrieval of membrane proteins from the Golgi apparatus to the ER. [63]
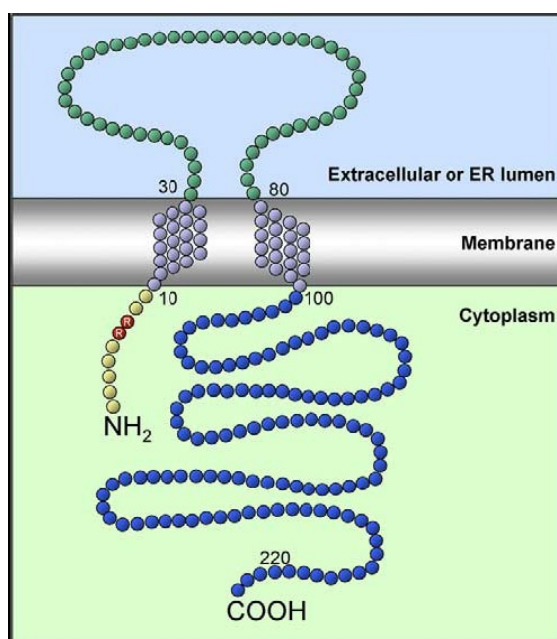

Figure 5: Sigma-1 receptor (Aydar and co., 2004)

Subcellular localization

The presence of a retention signal in sigma-1 sequence supports that ER is the main locus where sigma-1 exists. Immunofluorescence studies have demonstrated that sigma receptors

are predominant in cytoplasmic areas of neuronal and retinal cell bodies, indicating an ER localization.[64,65] Recently, Maurice and coworkers performed the first electron microscopic examination of sigma-1 in the adult animal, confirming that sigma-1 receptors are highly expressed in ER cistaernae. However sigma-1 receptors were also found in the limiting plasma membrane, in mitochondrial membrane and in postsynaptic thickening of neurons. [66]

Signal transduction mechanism and modulatory action

So far, the signal transduction mechanism of sigma-1 receptors is still not well clarified, but it is clear that different cell types respond to a different action mechanism.

Recently, the most prominent and the most explored molecular action of sigma-1 receptors regards their interaction with ion channels. In fact it was discovered that the overexpression of sigma-1 ER receptors, induced by a sigma agonist administration, could cause their translocation from the ER to the sub-plasma membrane area where they could interact with ion channels.[67] For instance, Hayashi and Su, proposed a model where the IP3 receptor-inhibiting protein, ankyrin, was removed from IP3 receptors when sigma-1 receptor agonists were applied to NG-108 cells, resulting in an enhancement of Ca^{2+} efflux from the ER into the cytosol.[68] Wu and Bowen (2008) observed the same results they found that the C-terminus portion of sigma-1 receptors caused the dissociation of ankyrin from IP3 receptors in MCF-7 tumor cells.[69]

Aside from the already discussed functions, where sigma-1 receptor is directly involved, it is also noteworthy its regulatory activity on a number of known neurotransmitter systems including modulation of dopamine and acetylcholine synthesis and release;[70,71,72] modulation of NMDA glutamate receptor electrophysiology;[73] modulation of NMDA-stimulated norepinephrine release;[74,75] modulation of

phosphoinositol-stimulated turnover of muscarinic receptor;[76] modulation of opioid analgesia;[77] neuroprotective and anti-amnesiac activity;[78] cocaine-induced locomotory alteration and toxicity.[79,80]

Selective sigma-1 ligands and pharmacophoric model

Recently several ligands with high sigma affinity have been synthetized. The phenylethylenediamine class is noteworthy because the ligand BD1008 and its derivatives have shown high affinity toward both sigma subtypes,[81, 82] in this case the affinity was enhanced by the substitution with different lipophilic substituents. Particularly interesting, was the substitution of a phenyl group with a benzomorphanic group, which conducted to a ligand with a high sigma 1 affinity (Ki < 10 nM) and lower sigma 2 and opioid affinity.[83]

Similar to the phenylethilendiamine class are the phenylpenthylamine series, in which the scaffold contains only one nitrogen atom that is sufficient to exert a consistent sigma-1 affinity (Ki = 1 nM). Moreover this scaffold was used to hypothesize a pharmacophoric model (Fig. 6), where the two binding hydrophobic regions have to be opportunely distanced (2.5-3.9 Å and 6-10 Å) from the nitrogen atom, which could be secondary, tertiary or quaternary.[84]

The introduction of an ester group on the five terms chain produced the AC915 that possesses a 2000 fold greater affinity for sigma-1 versus sigma-2.[85]

The modifications to the haloperidol structure have led to the synthesis of E-5842, a high selectivity sigma-1 ligand.[86] Regarding the selectivity significant results were obtained also with phenylacetamide class.[87]

Among the few synthesized antagonists, the Ne-100 possesses a high sigma-1 affinity and moderate selectivity toward sigma-2, dopaminergic D_2, serotoninergic 5-HT$_2$ and PCP receptors. It has also been proposed as an antipsychotic drug.[88]
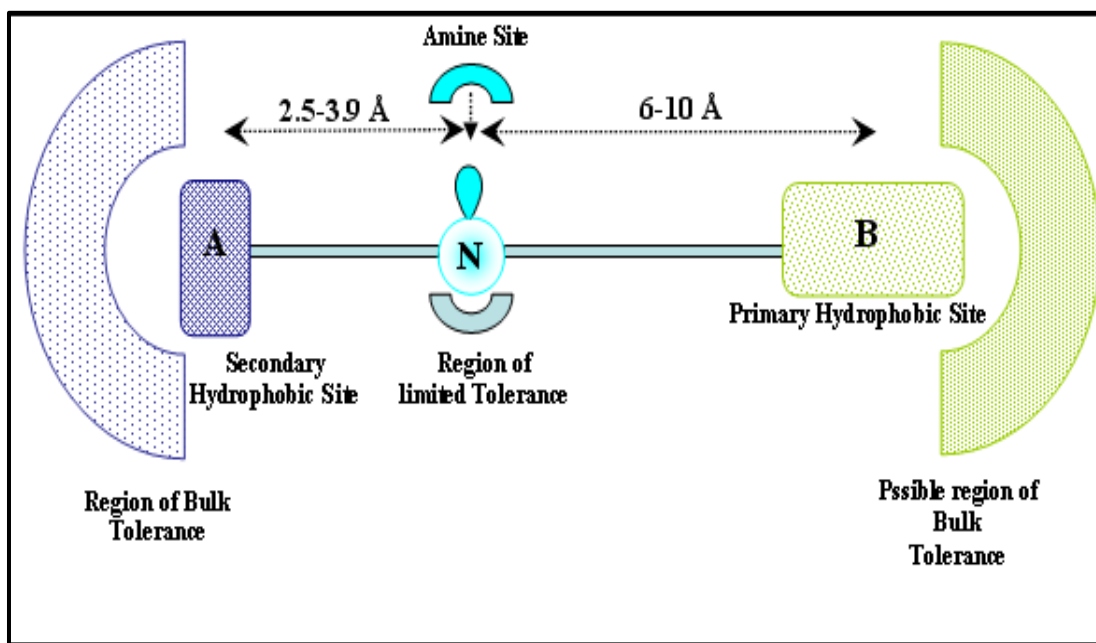
Figure 6: The sigma-1 pharmacophoric model of Ablordeppey-Glennon

Sigma-2 receptor

Characterization

Unlike sigma 1 subtype the sigma-2 is still not well characterized overall because it has not been cloned yet and there is no information about its sequence and conformation. Thanks to electrophoretic studies and photoaffinity labelling methods, it is known that the molecular weight of sigma-2 receptor is around 18-21.5 KDa, therefore it is smaller than sigma-1.[89]

Even the pharmacological characterization of sigma 2 receptors has been difficult and incomplete because of the lack of high selective sigma 2 radioligands. Usually [^{3}H]-DTG is used as radioligand in sigma-2 receptor binding assays, but it is also active for sigma-1 sites. As consequence, it is necessary to block sigma-1 sites with (+)-pentazocine or with dextrallorphan. On the basis of its incomplete pharmacological characterization it is possible to identify the sigma 2 site as a receptor with high affinity for haloperidol (table 3, Ki = 79.8 nm on rat brain) and DTG (table 3, Ki 61 nM on rat brain) but low affinity for (+)-benzomorphan derivatives, preferring the (−) stereoisomers.[89,15] The phenylenilmorphan CB-64D and CB-184, the alkaloid ibogaine and the ifenprodil have shown to possess a high sigma-2 affinity and selectivity. Unfortunately, they also have high affinity for other receptor systems: ibogaine and ifenprodil toward NMDA channel receptors and the phenylenilmorphans toward μ opioid receptors.[90,91,92]

The current challenge of many researchers is the development of new highly selective ligands for sigma-2 receptors. More importantly researchers have obtain tools able to properly study this subclass and to achieve a better understanding of its functions.

Subcellular localization

Early studies, regarding the subcellular localization, have shown the presence of sigma-2 and sigma-1 receptors in microsomal fractions (the richest in sigma receptors), mitochondrial and brain synaptosomal fractions of C57BL/6 mouse. In the microsomal and synaptosomal fractions the concentration ratio of the two subtypes resulted almost equal, whereas in the mitochondrial fraction the concentration of sigma-1 receptors was two times more than sigma 2 receptors.[93]

Unfortunately, the absence of a cloned sigma 2 gene and the lack of a purified receptor protein and related antibodies, have prevented researchers to study the sigma 2 subcellular localization through immunohistochemical techniques, as previously illustrated for sigma-1 receptor. However, recent development of highly selective sigma-2 receptor radioligands and fluorescent probes has been useful in the understanding of the subcellular distribution of sigma 2 receptor.

Bowen and co. (1996), during an attempt to purify the sigma receptor proteins, observed a low recovery of the solubilized sigma-2 receptor protein compared to sigma-1 receptor protein. They suggested a possible localization of sigma 2 receptors in detergent-resistant lipid raft domains.[16] Lipid raft are microdomains of the cell membrane, enriched with cholesterol, sphingolipids, and glycosylphosphatidylinositol-linked proteins that form specialized structures termed caveolin upon the incorporation of a cholesterol binding protein. Gebreselassie and Bowen, using sucrose density centrifugation of CHAPS[†] extracts of rat liver P2 membranes demonstrated that [^{3}H] DTG-binding site in protein fractions contained flotillin-2, a molecular marker of lipid rafts.[94] Subsequent in vitro binding studies revealed that [^{3}H]-DTG binding was blocked by the sigma-2 selective

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[†] Zwitterionic detergent: 20 mM of 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate

ligand CB-64D and not by the sigma-1 receptor ligand (+)-pentazocine, confirming that sigma 2 receptors are co-localized in lipid rafts. Other in vitro binding studies using [^{3}H]-DTG in the presence of (+)-pentazocine have shown that sigma-2 receptor binding sites are located in the mitochondria.[95]

Using the fluorescent probes SW107 and K05-138, Zeng and colleagues,[96] lately conducted a series of confocal and two photon microscopy studies that provided a clearer picture of the localization of sigma 2 receptors in breast tumor cells. These studies were performed in EMT-6 (mouse breast tumor) or MDA-MB-435 (metastatic human tumor) using a panel of tracker dyes. Using the fluorescent probe SW107, Zeng and co. indicated that sigma 2 receptors are localized in the mitochondria, lysosomes, endoplasmic reticulum and cytoplasmic membrane. Similar results were obtained with confocal imaging studies using the fluorescent probe, K05-138, also demonstrating that ~40% of the sigma 2 receptors were internalized by receptor-mediated endocytosis, while the remaining ~60 % was internalized by other mechanisms such as passive diffusion. The rapid internalization of sigma-2 receptors via endocytosis suggests that sigma 2 selective ligands might be useful as receptor-mediated probes for delivering cytotoxic agents to solid tumors.

Sigma-2 receptors and the regulation of motor function

Since sigma-2 receptors are highly expressed in the brain regions regulating posture and participating in motor control, it is reasonable to think about their important contributions in the regulation of motor functions.[21,22] Indeed it has been proven that microinjections of selective sigma ligands into motor regions of brain induced marked alteration in movement and posture. Microinjections of typical neuroleptics, as well as selective sigma 2 ligands, into the rat red nucleus induced acute dystonic reactions.[97] While microinjections of sigma ligands into the facial nucleus or spinal trigeminal nucleus oralis, determined oral facial

dyskinesias.[98] Unilateral microinjections of sigma ligands into the substanzia nigra resulted in contralateral circling.[99] The effects on motor behaviour and posture were described with a pharmacological profile generally corresponding to mediations by sigma 2 receptors.[99,100] Therefore, these results suggested the involvement of sigma 2 receptors in the side effects of typical antipsychotic drugs, particularly tardive dyskinesias and acute dystonias.[101,102]

Sigma-2 receptors and cell death

Some of the brain microinjection studies, described above, suggested that some sigma ligands might be neurotoxic. For example, the major haloperidol metabolite, the reduced haloperidol (potent sigma ligand) and the cyclohexane diamine, BD614, were able to cause extensive gliosis and loss of magnocellular neurons in and around the injection site.[103, 104]

Furthermore *in vivo* studies have shown that some ligands were cytotoxic to tumour cell lines of both neuronal (e.g., SK-N-SH neuroblastoma) and nonneural (e.g., C6 glioma) origin, as well as to primary cultures of rat central nervous system: cerebellar granule cells, cortical neurons, superior cervical ganglion cells. [105,106,107] Initially, sigma ligands caused damage to cell processes followed by a loss of these processes, assumption of a spherical shape (rounding) and detachment from the surface. The continue exposure to sigma ligands determined cell death, with a dose dependent effect (higher doses caused morphological changes and the subsequent cell death). Sigma 2 receptors were considered the most responsible for these effects. Indeed, sigma ligands binding both sigma-1 and sigma-2 sites, such as haloperidol, were active in evoking apoptosis, whereas sigma-1 selective ligands, such as (+)-pentazocine were inactive. Also compounds without a significant sigma affinity but having affinity for other receptors were not able to induce apoptosis.

The involvement in cell death of sigma 2 receptors was confirmed using the selective sigma-2 ligands, CB-64D and CB184 (Fig. 7), which resulted in a quite potent

cytotoxicity.[90] Thus, it was clear that chronic activation of sigma 2 receptors produced morphological changes and cell death. The type of cell death induced by sigma 2 ligands, in different cell types, was widely proved to be apoptotic.[108] [109] In fact, after treatment of SK-N-SH neuroblastoma cells or breast tumour cell lines with sigma agonists, such as CB-64D and CB184, the typical hallmarks of apoptosis were observed: inversion of phosphatidyl serine, DNA fragmentation and nuclear condensation. Similar results were observed using primary culture of rat cerebellar granular cells.[108]

The sigma-2 receptors determine apoptosis through a modulation of intracellular calcium, which was demonstrated in studies that used indo-1-loaded human SK-N-SH neuroblastoma cell. In these studies sigma ligands, from different structural classes, showed the ability to determine two different types of $[Ca^{++}]_i$ (intracellular calcium concentration) enhancement.[110,111] The role of sigma 2 receptors in the mediation of the effect on $[Ca^{++}]_i$ was clearly indicated by three considerations: the high affinity of the sigma-2 ligand CB-64D, the lower activity of CB-64L (levo isomer), and the very low activity of selective sigma-1 ligands, such as (+)-benzomorphans, (+)-pentazocine, (±)-SKF-10047 and dextrallorphan.[111] The two types of $[Ca^{++}]_i$ rise were distinguishable temporally and by sources.[111] In the first type sigma-2 ligands stimulated a rise in $[Ca^{++}]_i$ immediate, dose-dependent, and transient, through a release of calcium from endoplasmic reticulum. This transient rise occurred in the absence of extracellular calcium and was inhibited by the pretreatment of cells with thapsigargin, an intracellular inhibitor of ATPasi calcium pump. In the second type, the prolonged exposure of cells to sigma 2 ligands resulted in a latent and sustained rise of $[Ca^{++}]_{i,,}$ which was not affected by thapsigargin pretreatment. In this case sigma-2 ligands also induced a release of calcium from

mitochondria stores or from some other calcium store insensitive to thapsigargin (e.g. from Golgi apparatus).

Ostenfeld and co. obtained important results studying the cytotoxic effects of Lu 28-179, also known as siramesine, previously evaluated as a drug to treat anxiety and depression. Lu 28-179 has shown the capacity in killing tumor cells via a caspase-independent method, inducing cell death via a lysosomal leakage pathway and resulting in the formation of reactive oxygen species.[112]

Because sigma 2 receptors are overexpressed in rapid proliferation cells they could have an important role in the control of cell proliferation through the modulation of $[Ca^{++}]_i$.[113]
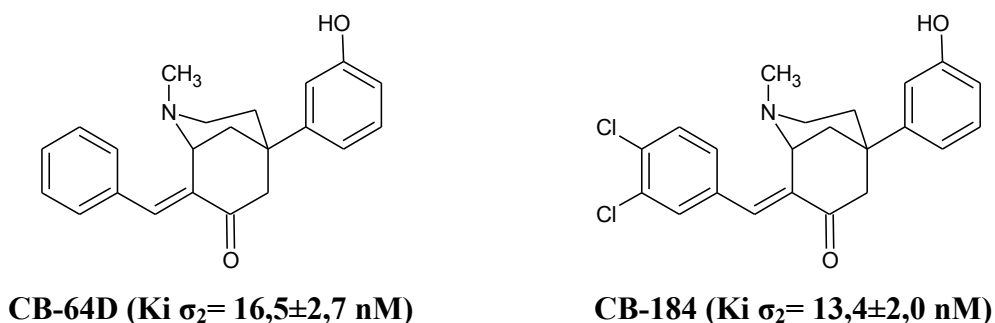

CB-64D (Ki σ$_2$= 16,5±2,7 nM)   **CB-184 (Ki σ$_2$= 13,4±2,0 nM)**

Figure 7: cytotoxic sigma 2 ligands

In a recent study two structurally distinct sigma-2 receptor ligands, SV119 and WC26 (Fig. 8), were found to induce apoptosis to mice and human pancreatic cancer cells *in vitro* and *in vivo* experiments.[114] Micro-PET imaging was used to demonstrate that the sigma-2 receptor was preferentially expressed in tumor tissues as opposed to normal tissues in pancreas tumor allograft-bearing mice. Sigma-2 receptor ligands, WC26 and SV119, and the sigma-1/sigma-2 promiscuous ligand haloperidol induced apoptosis in a dose

dependent fashion in all the pancreatic cell lines tested, with a caspase-3/7 dependent mechanism. The selective sigma-1 ligand pentazocine showed minimal toxicity toward all the tested cell lines. In vivo was observed that systemic administration of WC26 did not induce apoptosis in brain, lung, kidney or spleen at any of the concentrations tested. Pancreas and liver have shown to undergo a small amount of apoptosis (<10%) while their tumors had dose-dependent increases in apoptosis (up to 50% of tumor cells were active caspase-3 positive following a single 2 mg dose of WC26, $p < 0.0001$). The mice appeared normal and no apparent toxicity was noted in serum biochemistry data. Moreover WC26 significantly slowed tumor growth after a 5 day treatment compared to vehicle-injected control animals ($p < 0.0001$) and blood chemistry panels suggested a minimal peripheral toxicity.
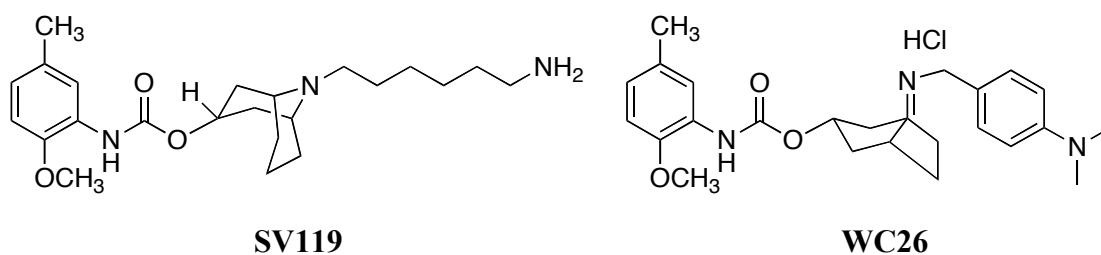

SV119　　　　　　　　　　　　　　　**WC26**

Figure 8: cytotoxic ligands

Sigma-2 ligands as probes for imaging in vitro and in vivo

Vilner et al.,[115] demonstrated that there is a higher density of sigma 2 versus sigma-1 receptors in a wide variety of human and murine tumor cells growing under cell culture conditions. An additional observation showed that MCF-7 cells, a human breast adenocarcinoma cell line, possessed a high density of sigma 2 receptors. The absence of

sigma-1, suggested that the sigma-2 receptor could be a potential biomarker for imaging studies of breast cancer.

Using the well-established diploid mouse mammary adenocarcinoma cell line 66, Mach and colleagues exposed that the density of sigma 2 receptors in 66P proliferative cells was about 10 times greater than the density observed in 66Q quiescent cells.[116] A subsequent study demonstrated that the upregulation and the downregulation of sigma 2 receptors follow the transition of mouse mammary cells between the proliferative and quiescent states. These data suggest that this receptor is not only expressed in a single phase of the cell cycle. [117]

Therefore, radiotracers having a high affinity and high selectivity for sigma-2 receptors have the potential to assess the proliferative status of human breast tumors using noninvasive imaging techniques such as Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT). This approach could be extended to assess the proliferative status of other human tumors, such as head and neck, melanoma, and lung tumors, which are known to express a high density of sigma-2 receptors.[118]

Although [³H]-DTG has been a very useful ligand for characterizing the sigma 2 receptor, and for identifying sigma 2 selective ligands, its rapid dissociation rate is not ideal for *in vitro* binding studies. Scatchard studies in rat and human brain samples revealed that [³H]Lu 28-179 (Fig. 9) has a Kd value of 1.1 nM.[119] Autoradiography studies of [³H] Lu 28-179 revealed a high density of sigma-2 receptors in the motor cortex, hippocampus, and hind brain nuclei, which was consistent with previous autoradiography studies using [³H] DTG in the presence of 1 nM dextrallorphan to mask sigma-1 binding sites. Other sigma 2 selective ligands that have been labeled with tritium include [³H]RHM-1,[120] and [³H]PB28 (Fig. 9).[121] [³H]-RHM-1 has the highest selectivity for

sigma-2 versus sigma-1 receptors.

A number of sigma receptor ligands have also been radiolabeled with iodine-125, a useful radionuclide in vitro binding studies. However, most ^{125}I-labeled ligands have a high affinity for the sigma-1 and low for the sigma-2 receptor. The only sigma-2 selective ligand labeled with iodine-125 reported is the conformationally-flexible benzamide analog in figure 9. [122,123,124]

In vivo, the conformationally-flexible benzamide analogs have proven to be the most useful in the development of PET radiotracers for imaging the sigma 2 receptor status of solid tumors.[125] MicroPET and MicroCT imaging studies in a murine solid breast tumor EMT-6 have shown the potential of the [^{11}C]-benzamide analogs (Fig. 9), as radiotracers for imaging the sigma 2 receptor status of breast tumors with PET.[126] The short half life of carbon 11 ($t1/2 = 20.4$ min) is not ideal for the development of radiotracers, thus benzamide analogs have also served as lead compounds in the development of ^{18}F-labeled sigma 2 selective radiotracers with a longer half life ($t1/2 = 109.8$ min). The strategy chosen involved replacement of the 2-methoxy group in the benzamide ring with a 2-fluoroethoxy group. MicroPET imaging studies indicate that [^{18}F] benzamides analogs, are suitable probes for imaging the sigma 2 receptor status of solid tumors.[127] Clinical studies of [^{18}F] benzamide analog in figure 9 are currently in progress in the U.S.

As discussed above, fluorescent probes SW107 and K05-138 (Fig. 9) have produced important results in the study of subcellular distribution of sigma receptors with two-photon and confocal microscopy.[96]
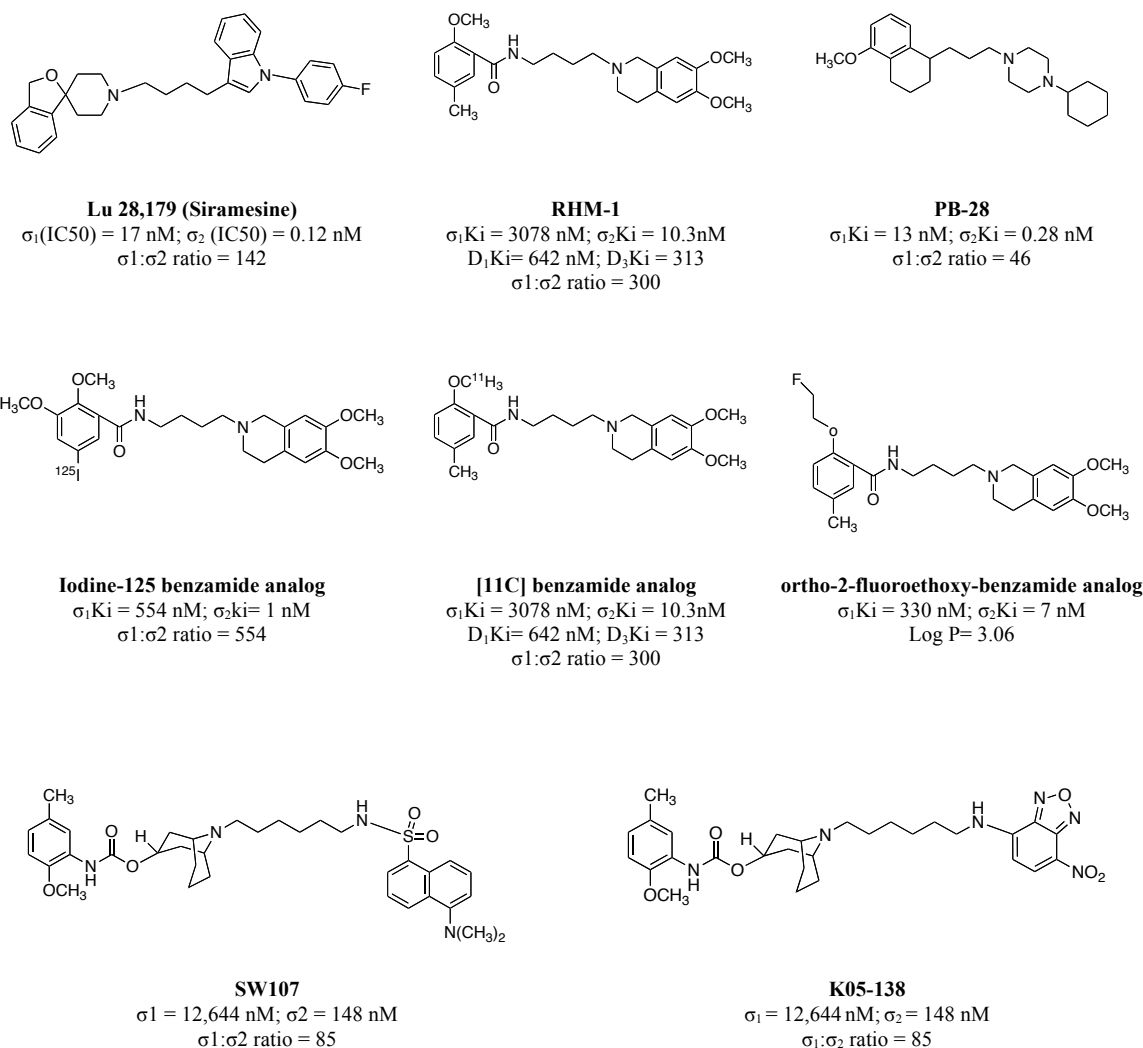
Lu 28,179 (Siramesine)
σ_1(IC50) = 17 nM; σ_2 (IC50) = 0.12 nM
σ1:σ2 ratio = 142

RHM-1
σ_1Ki = 3078 nM; σ_2Ki = 10.3nM
D_1Ki= 642 nM; D_3Ki = 313
σ1:σ2 ratio = 300

PB-28
σ_1Ki = 13 nM; σ_2Ki = 0.28 nM
σ1:σ2 ratio = 46

Iodine-125 benzamide analog
σ_1Ki = 554 nM; $\sigma_2$ki= 1 nM
σ1:σ2 ratio = 554

[11C] benzamide analog
σ_1Ki = 3078 nM; σ_2Ki = 10.3nM
D_1Ki= 642 nM; D_3Ki = 313
σ1:σ2 ratio = 300

ortho-2-fluoroethoxy-benzamide analog
σ_1Ki = 330 nM; σ_2Ki = 7 nM
Log P= 3.06

SW107
σ1 = 12,644 nM; σ2 = 148 nM
σ1:σ2 ratio = 85

K05-138
σ_1 = 12,644 nM; σ_2 = 148 nM
σ_1:σ_2 ratio = 85

Figure 9: sigma 2 ligands as probes for imaging

Sigma-2 ligands

The development of ligands having a high affinity for the sigma 2 versus the sigma 1 receptor has not been straightforward; unfortunately at the moment only a limited number of selective sigma-2 compounds are available.

The most pharmacologically studied sigma-2 ligands are the already cited benzilidene phenilmorphan analogs, CB-64D and CB-184 (Fig. 7). The benzomorphan-7-one analog, CB-64D, was the first selective sigma 2 ligand and it was identified during a structure-activity relationship (SAR) study on ligands of µ opioid receptors.[90,128] It was

observed that the adding of an (E)-benzylidene moiety into the 8-position of (−)-2-methyl-5-(3-hydroxyphenyl)morphan-7-one ring system increased affinity for sigma receptors. Specifically, the (−)-1S,5R isomer, CB-64L, showed a high affinity for sigma-1 versus sigma-2 receptors, whereas the (+)-1R,5R isomer, CB-64D, had a 185-fold higher selectivity for sigma 2 versus sigma 1 receptors. Particularly interesting was the profile of the corresponding 3,4-dichloro analog, CB-184, that was the ligand with the highest sigma-2 affinity and selectivity among those of the same series. The negative aspect of the phenylmorphan series is the interaction with the opioid receptors as shown by their high μ agonist activity *in vivo*, justified by the presence of the morphinanic moiety.[90]

Another important series of compounds having high affinity for sigma-2 receptors are the 3-(ω- aminoalkyl)-1H-indole analogs.[129,130] The optimal compound of these series was the 1'-[4-[1-(4-fluorophenyl)-1H-indol-3-yl]-1-butyl]spiro[isobenzofuran-1(3H),4'-piperidine], Lu 28-179 (Fig. 9), with a 17nM (IC50) affinity on sigma 1 receptor a 0.12 nM (IC50) on sigma 2 receptor. Furthermore it exerted a very low affinity on 5-HT1A and 5-HT2A (IC50: 21,000 nM and 2000 nM respectively) and moderate affinity on D_2 and alpha-1 receptors (IC50: 800 nM and 330 nM respectively). However, the most selective sigma 2 versus sigma 1 ligand of this class was the tropane derivative 1-(4-fluorophenyl)-3-[4-[3-(4-fluorophenyl)-8-azabicyclo[3.2.1]oct-2-en-8-yl]-1-butyl]-1H-indole with a 1200 nM (IC50) affinity for sigma 1 receptor and 2.5 nM (IC50) affinity for sigma 2 receptor.

A natural compound that was found to have a high affinity for sigma 2 versus sigma 1 receptors is the hallucinogen, ibogaine, which binding studies and SAR will be discussed afterwards in the text. [131] Both CB-184 and ibogaine contain in their structure an arylpropylamine moiety that is a useful requirement for sigma-2 receptor affinity, whereas compounds with affinity for sigma-1 sites, such as Ne 100, tend to possess

phenylethylamine moiety.[132] This was demonstrated by the phenylpropylpiperidine in figure 9 that has an affinity 4 fold higher for sigma-2 versus sigma-1. The modifications on the amine group led to significant increasing in sigma-2 affinity, as shown by the arylpropylpiperazine derivative in figure 10.[132]
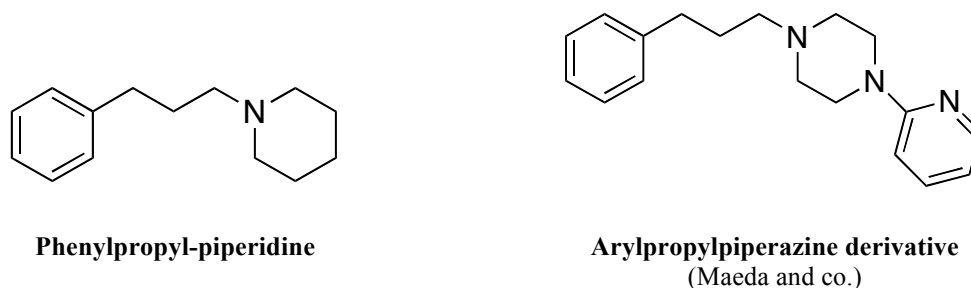

Phenylpropyl-piperidine   **Arylpropylpiperazine derivative**
(Maeda and co.)

Figure 10: sigma 2 ligands of Arylpropylamine series

Instead, Berardi and co. studied a very promising series of substituted tetralines containing a gem-dimethyl piperidine (Fig. 11).[133,134] In these series they observed that when the aliphatic chain was 4 terms long the derivative showed a very high sigma 2 affinity ($\sigma_2 IC_{50}$ = 0.016 nM) and a 100,000 fold higher selectivity for sigma 2 versus sigma-1 receptor. Interestingly, in the derivative with 5 terms chain ($\sigma_2 IC_{50}$ = 0.016 nM) the selectivity diminished to 21 fold. The derivative without a methoxy group and with a 5 terms chain (des-methoxy, n = 5, $\sigma_2 IC_{50}$ = 0.008 nM) showed again a 100,000-fold higher selectivity for sigma-2 versus sigma-1.

Kawamura and co-workers, highlighted that small structural differences have a great impact on the sigma 2 affinity and selectivity.[135] For example in the compound in figure 11 when R is a methyl group the derivative binds sigma-2 receptor with low affinity (K_i = 1800 nM) and possesses a 106-fold higher selectivity for sigma 2 versus sigma 1. When R

is an ethyl group, the derivative binds sigma-2 receptor with high affinity (Ki = 13 nM) but the selectivity is lower (σ1:σ2 ratio to 3).

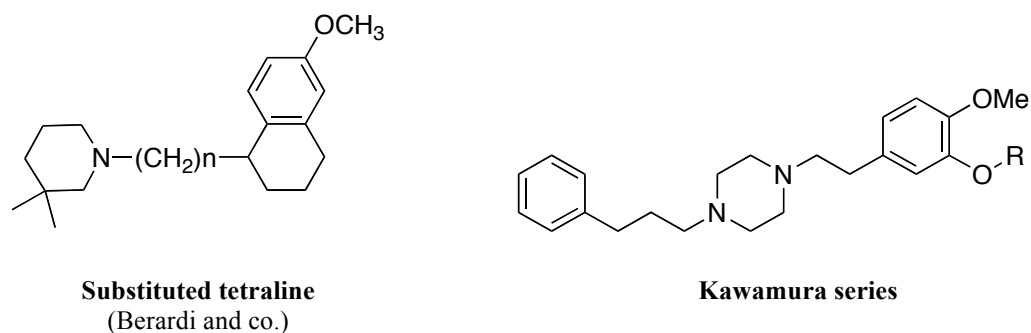**Substituted tetraline**
(Berardi and co.)

Kawamura series

Figure 11: sigma 2 ligands

New sigma-2 ligands containig a tropane nucleus have shown to possess a sigma-2 affinity up to 5 nM and a 500-fold higher selectivity for sigma 2 versus sigma-1. The addition of an amine group in para position to the benzene ring was showed useful in increasing the sigma 2 selectivity of the un-substituted analogue (Fig. 12).[136] An important tropane analogue is the ($\pm$)-SM-21 (Fig. 12) that was used as antagonist in behavioural assays where it was able to reduce the convulsive and locomotory effects of cocaine. Although early studies showed a significant sigma 2 affinity and around 14 selectivity for sigma 2 versus sigma-1 (rat liver σ_1Ki > 1000 and σ_2Ki = 67,5$\pm$8) subsequent studies showed a lower sigma 2 affinity (guinea pig σ_2Ki = 434 nM; σ_1Ki >1000 nM), most likely because a different tissue was used in the later binding study. [137,138,139]


Tropane nucleus ligand
(Mach and co.)

($\pm$)-SM-21
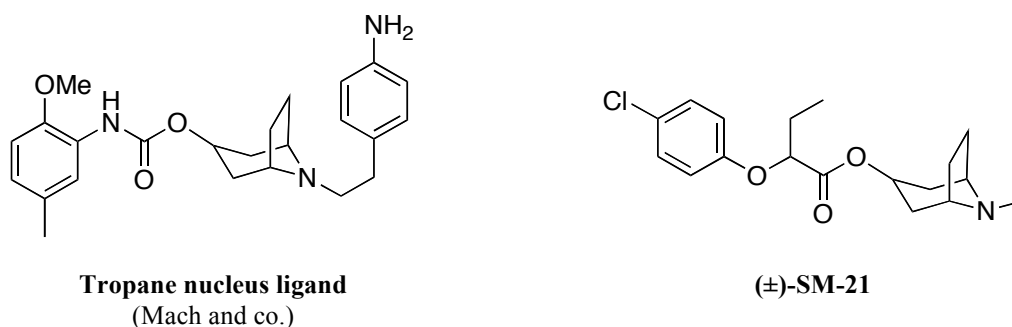
Figure 12: Tropane nucleus ligands

Several SAR studies on **BIMU-1 (**32 nM σ_2 affinity; 6,300 nM σ_1 affinity) (Fig. 13) as the lead compound have conducted the identification of high affinity and high selectivity sigma 2 receptor ligands.[140,141,142] Addition of a benzene ring to the N-methyl of the bridgehead nitrogen, giving the corresponding N-benzyl group, and the replacement of the urea linkage of BIMU-1 resulted in a significant increase in affinity for both sigma 2 and sigma-1 receptors, a loss of affinity for 5-HT3 receptors and moderate affinity for 5-HT4 receptors.[140] Expansion of the tropane ring to the corresponding granatane ring eliminated affinity for the 5-HT4 receptor and did not change the affinity for sigma-1 and sigma-2 receptors, relative to the tropane analogs. The most interesting analog from this initial SAR study was the granatane derivative in figure 13, which had a sigma 2 receptor affinity of ~ 3 nM and a sigma 2 versus sigma 1 selectivity of ~30. [140]

Using this granatane analog as lead compound a further SAR study was started and it was observed that the substitution of benzyl group with a 2-phenethyl group resulted in a slight improvement in sigma 2 receptor affinity and increased sigma-2 selectivity (σ1:σ2 ratio to ~50).[141]Substitution in para position to the aromatic ring of the granatane lead compound with a dimethylamino group resulted in a further increase in the selectivity for sigma 2 receptors, because of the reduction in affinity for sigma 1 receptors.[141]The amino aminoalkyl group also appeared as a good substituent for assuring a high affinity for sigma-2 receptors and high sigma-1/sigma-2 selectivity ratio.[142] These results were used in the design of the fluorescent probes SW107 and K05-138 (Fig. 9), useful tools in two photon and confocal microscopy studies of sigma 2 receptors (already described in the last section).
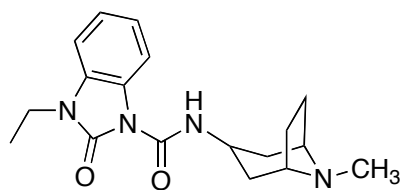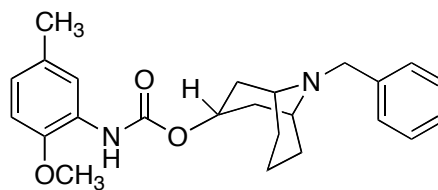
BIMU-1   **Granatane analog**

Figure 13: Sigma 2 ligands based on tropane and granatane analogs

Another class of compounds having a high affinity for sigma 2 receptors and excellent sigma-1/sigma-2 selectivity ratios are the conformationally-flexible benzamide analogs (Fig. 14). The analog with a 6,7-dimethoxy-1,2,3,4-tetrahydroisoqinoline ring (e.g. Fig. 14 on the left) resulted in compounds having a high affinity, excellent selectivity for sigma-2 versus sigma-1 receptors and a dramatic reduction in affinity for dopamine receptors, relative to its lead compound. [143] The removal of 6,7-dimethoxy group was critical for sigma 2 receptors affinity. Replacement of the 5-bromo group with a methyl group (Fig. 14 on the right) resulted in a further reduction in D_3 receptor affinity and no change in affinity for the sigma-2 receptor. Extension of the 2 carbons spacer to the corresponding 4 carbons spacer, of RHM-1 (Fig. 9), also retained a high affinity for sigma 2 receptors. RHM-1 as discussed above was used successfully as a radio-labeled probe for sigma 2 receptors.
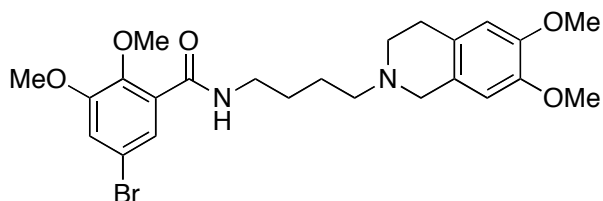
Analog with a 4 carbons spacer
$\sigma_1 = 3{,}078$ nM; $\sigma_2 = 10.3$ nM; $\sigma_1{:}\sigma_2$ ratio = 300
$D_3 = 627$ nM; $D_2 = 2{,}200$ nM
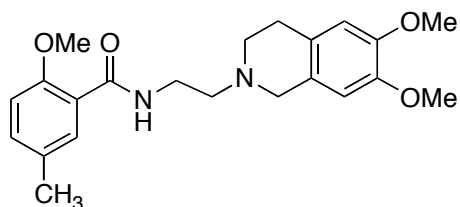
Analog with a 2 carbons spacer
$\sigma_1 = 10{,}400$ nM; $\sigma_2 = 13.3$ nM; $\sigma_1{:}\sigma_2$ ratio = 780
$D_3 = 3{,}760$ nM; $D_2 = 2{,}850$ nM

Figure 14: Examples of conformationally-flexible benzamide analogs with Ki values.

Proposed pharmacophoric model for sigma-2 receptor

Recently, Laurini E. *et al.,* developed a 3D-pharmacophore model for sigma 2 receptors based on a series of substituted benzo[d]oxazol-2(3H)-one derivatives.[144] The best output hypothesis (Fig. 15) contained a positive ionizable atom (PI), a hydrogen bond acceptor group (HBA), a hydrophobic aromatic site (HYAr), a hydrophobic aliphatic site (HYAl), and a generic hydrophobic site (HY).

The proposed pharmacophore model for the sigma-2 receptor showed remarkable similarities but also some differences with the validated 3D-QSAR developed, by the same authors, for sigma-1 receptors. A basic amino nitrogen atom between two hydrophobic sites is pharmacophoric element required for affinity. In the sigma-2 receptors the positions and distances between these features are different with respect to the sigma 1 receptor model. The primary hydrophobic site corresponds with the hydrophobic aromatic sphere (HYAr), which is mapped by the phenyl group connected to the aliphatic spacer, at a distance of 3.96 Å from the positive ionizable feature of the amino group, in agreement with their sigma-1 receptor model (3.58 Å),[145] Laggner's model (4.1 Å),[146] and the optimum distance suggested by Glennon et al. (2.5–3.9 Å).[147] Whereas, the secondary hydrophobic site is matched by the generic hydrophobic sphere (HY) that maps to the aromatic ring of the benzooxazolone group at a shorter distance (4.96 Å) with respect to the sigma-1 receptor model (8.50 Å). The generic hydrophobic feature (HY) is mapped by the aromatic ring of the benzooxazolone in sigma-2 hypothesis. The presence of the hydrophobic aliphatic feature (HYAl) constitutes a necessary requisite for binding with high affinity to sigma-2 receptors, while it does not seem to play a major role for selectivity towards this receptor. The hydrogen bond acceptor feature (HBA) corresponds to the carbonyl oxygen of the benzooxazolone moiety.
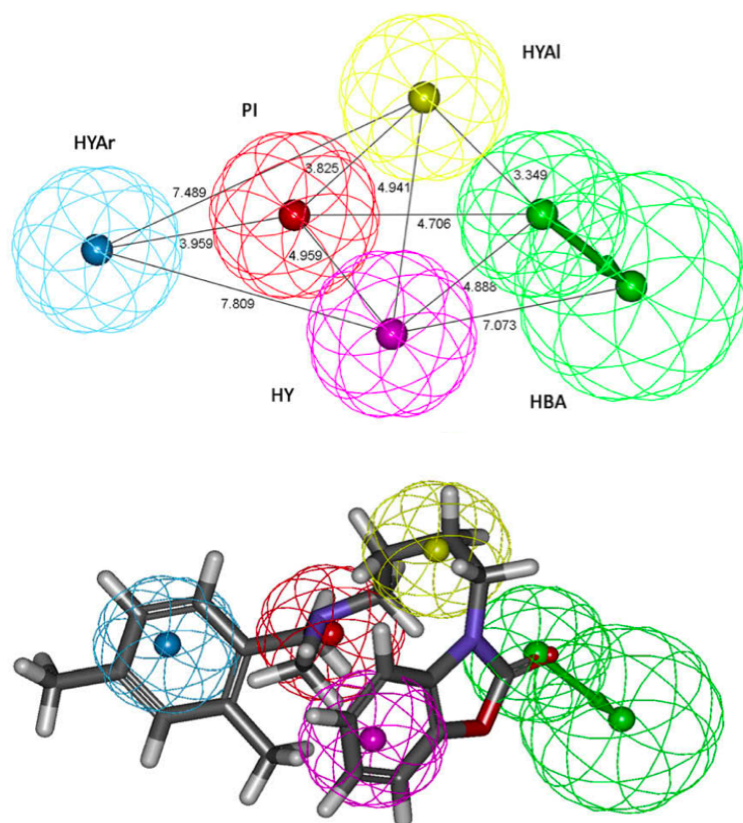
33

Figure 15: Top-scoring pharmacophore model Hypo1, based on a series of substituted benzo[d]oxazol-2(3H)-one derivatives, and mapping of a compound belonging to the same series. The hypothesis features are portrayed as mashed spheres, color-coded as follows: red, PI; light blue, HYAr; pink, HY; light green, HBA; yellow, HYAl. HBA is actually represented as a pair of spheres (the smaller sphere represents the location of the HBA atom on the ligand and the larger one the location of an HB donor on the receptor). Selected distances (Å) are labeled. Compounds are portrayed as atom-colored sticks (red, O; gray, C; blue, N; white, H).[144]

Ibogaine: pharmacological profile

Ibogaine (trade name: Endabuse ™), an indolic alkaloid, is contained in a number of plants but principally isolated from the shrub *Tabernanthe iboga* of central Africa. Ibogaine, as demonstrated by *in vivo* studies, is a psychoactive substance that exerts stimulatory effects on CNS (central nervous system), if administrated in low doses, whereas induces hallucinogen effects and tremors when administrated in high doses.[148,149]
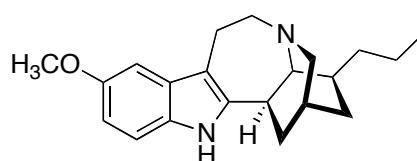

Figure 16: ibogaine

Because of its psychotropic effects ibogaine was used in the past as an abused drug. Anecdotal reports suggested that ibogaine was able to interrupt the craving of heroine, cocaine and amphetamines in addicts.[150] These observations were supported in several animal studies where ibogaine has been shown to reduce self-administration of both morphine and cocaine. [151,152,153,154] Unfortunately, the clinical use of ibogaine has found many troubles related to its neurotoxic and tremorigen effects. Specifically the treatment of rats with ibogaine 100mg/Kg determined microglia and astrocytes activation and loss of Purkinje cells in the parasagittal zones of cerebellum.[155,156]

The receptor sites responsible for the beneficial effects (anti-addiction) of ibogaine are not well known, but it was hypothesized that they are the result of a multiple low affinity interaction with several receptor systems. In vitro studies have showed that ibogaine possessed a micromolar affinity for σ_1 receptors; μ, κ and δ opioid receptors; M1, M2 and M3, muscarinic receptors; D_1 and D_2 dopaminergic receptors; GABA A site and

benzodiazepine site in GABA receptor; 5-HT2A e 5-HT3, serotoninergic receptors; α1, α2 and β adrenergic receptors; and for PCP site in NMDA channel. In contrast ibogaine has showed a nanomolar affinity for σ_2 receptor (Ki=250 nM in guinea pig brain, Ki = 90 nM in rat liver) and higher than 100-fold selectivity for sigma 2 versus sigma 1 (table 5). [157]

Table 5. Affinities of ibogaine, haloperidol, haloperidol metabolite II for sigma receptors.

Compound	σ_1[b]			σ_2(liver)[c]			σ_2(brain)[d]			σ_1 / σ_2 ratio
	K_i [nM] (n = 3) [a]									
Ibogaine	9310	±	630	90.04	±	10.1[e]	250	±	39[f]	103[g] or 37.2[h]
Haloperidol	1,77	±	0.09	21.8	±	8.5	14.2	±	3.2	0.08[g] or 0.13[h]
Haloperidol metabolite II	5.65	±	0.26	25.4	±	3.3	1.31	±	0.03	0.22[g] or 4.30[h]

[a] Mean ± SEM. [b]K_i for inhibiting the binding of [³H](+)-pentazocine to guinea pig brain membranes. [c]K_i for inhibiting the binding of [³H]DTG to rat liver membranes. [d]K_i for inhibiting the binding of [³H]DTG to guinea pig brain membranes. [e]N = 4. [f]N = 7. [g]K_i for σ_1 / K_i for σ_2 (liver). [h]K_i for σ_1 / K_i for σ_2(brain).

When Bowen W. D analysed the pharmacological profile of ibogaine, he hypothesized that the anti-addiction effects of ibogaine were not related to the high sigma-2 affinity but probably due to the interactions with other receptor systems. On the contrary sigma-2 receptors could be responsible of the ibogaine-induced neurotoxicity and tremorigen effects. [158] These considerations were supported by data showing the ability of ibogaine to induce apoptosis in nervous cells, related to the increase of $[Ca^{2+}]_i$, and by the ability of sigma 2 ligands in inducing motor alteration, as already discussed (see the section: sigma 2 receptor and the regulation of motor function). Actually, the cytotoxicity in humans seems attenuated by the metabolism of ibogaine that determines the *O*-demethylation to noribogaine,[159,160] which lacks affinity for sigma-2 receptors and produces no effects on $[Ca^{2+}]_i$ and no cytotoxicity *in vitro*.[158]

Ibogaine and its related alkaloids: SAR

Bowen and co. studied ibogaine and its related alkaloids with binding assays at sigma 1 and sigma 2 receptors and the data are summarized in table 6.[161] Sigma-1 receptors, in guinea pig brain membranes were labeled with the sigma-1 selective probe, [3H](+)-pentazocine, while sigma-2 receptors in rat liver membranes were labeled with [3H]DTG in the presence of dextrallorphan to inhibit the binding to sigma-1 sites. Ibogaine exhibited moderate affinity for sigma-2 sites (Ki = 201 ± 24 nM), but had very low affinity for sigma-1 receptors (Ki = 8,554 ± 1,134 nM), resulting in 43-fold selectivity for sigma-2 sites over sigma-1. Mach and co., as previously showed, obtained similar results with the ibogaine binding at sigma receptors in guinea pig brain, whereas using rat liver the data showed a higher selectivity for sigma-2 (table 5: σ_1 / σ_2 ratio to 103).[157]

Regarding the structure-activity relationships of iboga derivatives for affinity at sigma receptors (table 6),[161] (±)-Ibogamine, considered as parent compound, has an unsubstituted indole moiety, with a sigma-2 Ki = 137 ± 13 nM and sigma 1 Ki = 1,835 ± 131 nM. Thus, the methoxy group in the 10-position (ibogaine) did not significantly change the sigma-2 affinity, but decreased the sigma-1 affinity (Ki = 8,554 ± 1,134 nM). A methoxy group in the 11-position (tabernanthine) produced no significant change in sigma-2 affinity and a small decrease in sigma-1 affinity (Ki = 2,872 ± 37 nM), resulting in 14.8-fold selectivity for sigma-2 receptors. Alike an *O-t*-butyl group in the 10-position did not dramatically change the sigma-2 receptor affinity or the sigma-1 affinity (Ki = 4,859 ± 682 nM), resulting in 20-fold selectivity for sigma-2 sites. A hydroxyl group in the 10-position (noribogaine) resulted in a 38-fold loss of binding affinity at sigma- 2 receptors and 8-fold loss of affinity at sigma-1 receptors (Ki = 15,006 ± 898 nM). Thus, the sigma-2 binding site did not tolerate the phenolic hydroxyl group. The presence of a carbomethoxy group in

the 16-position ((±)-coronaridine) determined complete loss of sigma-2 affinity and a 20-fold loss in sigma 1 affinity (Ki = 35,688 ± 2,858 nM) in comparison to (±)-ibogamine. Addition of a methoxy group at the 18-position of the 16-carbomethoxy analog, (±)-18-methoxycoronaridine ((±)-MC), increased the sigma-2 affinity compared to (±)-coronaridine, but still had low affinity. (±)-MC had slightly improved sigma-1 binding affinity (Ki = 28,687 ± 283 nM) compared to (±)-coronaridine.

Thus, ibogaine can be considered as a ligand with good selectivity for sigma 2 receptor over sigma-1 that could be used as a model in designing new sigma-2 agonists and sigma 2 antagonists.

Table 6. Affinities of ibogaine and related indole alkaloids at sigma 2 receptors.[a]

Alkaloid	R_1	R_2	R_3	R_4	σ_2 K_i nM[b]	σ_1 K_i nM[c]
(±)-Ibogamine	H	H	H	H	137 ± 13	1,835 ± 131
Ibogaine	OCH_3	H	H	H	201 ± 24	8,554 ± 1,134
Tabernanthine	H	OCH_3	H	H	194 ± 10	2,872 ± 37
10-t-Butoxy-Ibogamine	O-t-Bu	H	H	H	247 ± 27	4,859 ± 682
Noribogaine	OH	H	H	H	5,266 ± 1,426	15,066 ± 898
(±)-Coronaridine	H	H	CO_2CH_3	H	>100,000	35,688 ± 2,858
(±)-MC	H	H	CO_2CH_3	OCH_3	8,472 ± 1,237	28,687 ± 283

[a]Portions adapted from data of Bowen and co.[161] [b]Sigma-2 receptors were labeled with [3H]DTG using rat liver membranes, in the presence of dextrallorphan to inhibit binding to sigma-1 sites. [c]Sigma 1 receptors were labeled with the sigma-1-selective probe, [3H](+)-pentazocine, in guinea pig brain membranes.

Aims of the work and drug design

Focusing my research work on natural scaffolds possessing sigma 2 affinity, I have chosen the alkaloid ibogaine (Fig. 16) as reference compound of a new series of sigma-2 ligands. Although ibogaine has moderate affinity for sigma 2 receptors (Ki ≈ 250nM in guinea pig brain or Ki ≈ 90 in rat liver) it has a significant selectivity for sigma-2 versus sigma-1 (σ_1 / σ_2 ratio to 103 in rat liver).[157] Therefore the main aim of the work was to design and synthesize new alkaloid-based ligands possessing an improved sigma-2 affinity and better or maintained sigma-2 selectivity with respect to ibogaine.

Individuation of a natural scaffold

I carried out a thorough literary investigation in order to find a natural scaffold containing the structural requirements necessary for sigma-2 receptor recognition and possessing a structure as similar to ibogaine as possible. Among the several indolic nucleus alkaloids considered, I observed that vinca-based alkaloids seem to possess the right pharmacophoric features for my purposes.

The eburnamine-vincamine alkaloids occur in the Apocynaceae plant family. The group can be divided into three major subgroups: 1. (−)-eburnamine; 2. (+)-vincamine; 3. strempeliopine. The five ring system is characteristic of these alkaloids. The "eburna" skeleton with the (20R, 21R) [(20β, 21β)] configuration belongs to the compounds termed eburnane types. Compounds with the (20S, 21S) [(20α, 21α)] configuration ("vinca" skeleton) belong to the vincane type of compunds (Fig. 17).[162] Vincamine, the main alkaloid isolated from the leaves of *Vinca minor*,[163,164] is the mother compound of cerebrally active eburnamine derivatives. Vincamine, as well as vincanol, is a potent

blocker of the voltage-gated sodium channels.[165] Vincamine reduced [³H]batrachotoxin binding (IC50 1.9 μM) in rat cortical synaptosomes, blocked the voltage-gated sodium current (IC50: 40 μM) in voltage-clamped rat cortical neurons and protected against veratridine induced cell death (IC50: 26) in cortical cultures. Vincamine and its related alkaloids (Fig. 17) including (–)-Eburnamonine (or vincamone), vindeburnol, vinpoceptine have shown modulatory effects on brain circulation and neuronal homeostasis, bearing antihypoxic and neuriprotective potencies to various degree.[162,166,167] Also Vincane is pharmacologically known for its marked vasodilatatory effect (5%-10%) in cerebral blood vessels.[168]

	Vincane: R^1, R^2 = H
	Vincamine: R^1 = COOCH₃; R^2 = OH
	(–)-eburnamonine (or vincamone): R = O (lactamic carbonyl at position 16)
	Vinpoceptine: R^1 = COOCH₂CH₃; R^2 = H; double bond at positions 16-17
	Vincanol: R^1 = OH, R^2 = H
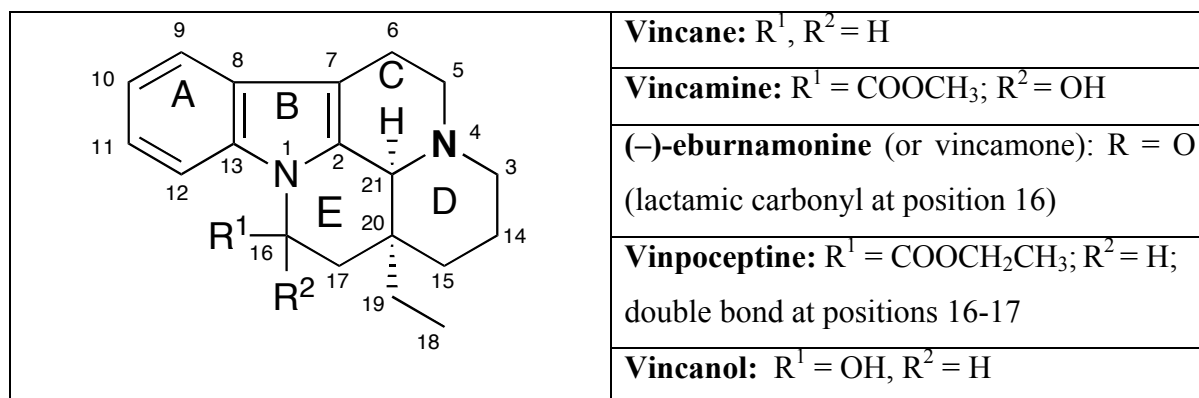
Figure 17: vinca skeleton and vincane type alkaloids

Phenylpropylamine moiety

N Tertiary Nitrogen
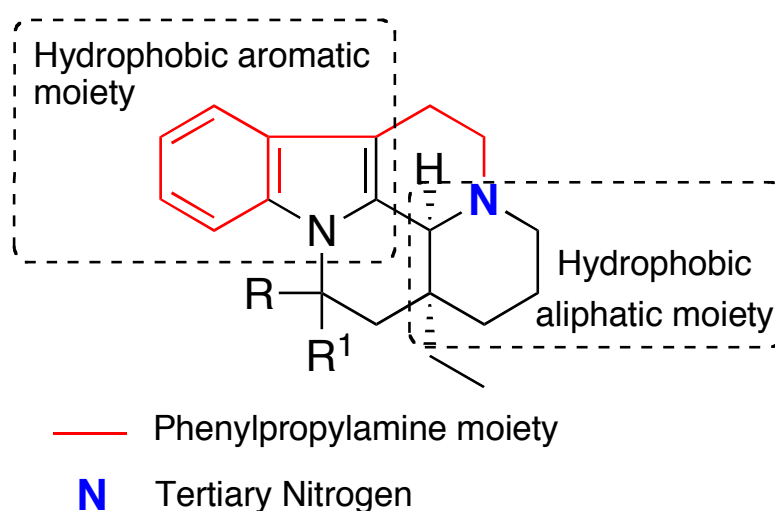
Figure 18: Structural requirements of vinca-scaffold

Considering the general structure of vincane type alkaloids (Fig. 17) it is possible to recognize four crucial requisites for the interaction with sigma-2 receptor. A positive ionisable atom is present, as described in all the sigma pharmacophoric models,[144,146,147] and corresponds to the tertiary nitrogen (in blue in Fig. 18). A phenylpropylamine moiety, highlighted in red in figure 18, as in ibogaine, CB-184 and arylpropylpiperazine could be useful for sigma 2 selectivity.[132] The vinca-scaffold contains a hydrophobic aromatic moiety corresponding to the indolic ring (Fig. 18), that should interact with the primary hydrophobic region of sigma-2 receptor, as well as the pharmacophoric model suggest.[144] Finally, into the vinca scaffold it is possible to individuate a hydrophobic aliphatic moiety corresponding to the D ring (Fig. 17), as well as the piperidine ring in several ligands, that should be necessary for binding with high affinity to sigma 2 receptors (Fig. 18).

Superimposition study: vinca-derivatives upon ibogaine

The pharmacophoric considerations, discussed in the last section, were supported by a computational superimposition study where the vinca scaffold was overlaid on the ibogaine structure. The study was carried out using the software MOE (Molecular Operating Enviroment) Chemical Computing Group. Thus, I have considered the selective sigma-2 ligand ibogaine as reference compound and Vincamone and Vincane as compounds containing the scaffold. The three structures were first minimized to the lower energy of the system and subsequently overlaid, using force field MMFF94X, at standard conditions[‡] and with preserved chirality (Fig. 19, 20). The superimposition resulted in an optimal structural homology of both vincamone and vincane with ibogaine (Fig. 20).


[‡] Gradiente 0.00001; flexible alignment: iteration limit 500; failure limit 30; energy cutoff 15; minimization gradient 0.00001; preserved chirality.

In both superimpositions it is possible to observe complete co-planarity of the two indolic nuclei. The tertiary nitrogen (labelled in blue) occupies the same position in both iboga-structure and in vinca-structures. The ring termed D of vinca scaffold (in Fig. 17) is lifted in respect to the indole plane and seems to occupy the same volume and position of the aliphatic bicycle in ibogaine. Finally, the ethyl chain of both structures seems correctly positioned.
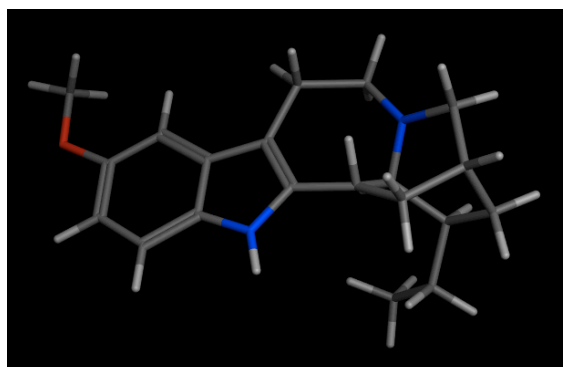
The main difference that occurs in vinca scaffold respect to ibogaine structure is the presence of the ring termed E (Fig. 17), in which is contained the indolic nitrogen. It would be interesting to extrapolate from binding assays information about the tolerance of sigma-2 receptor about this structural difference.
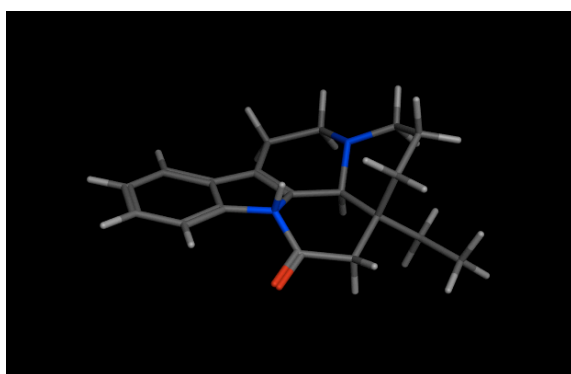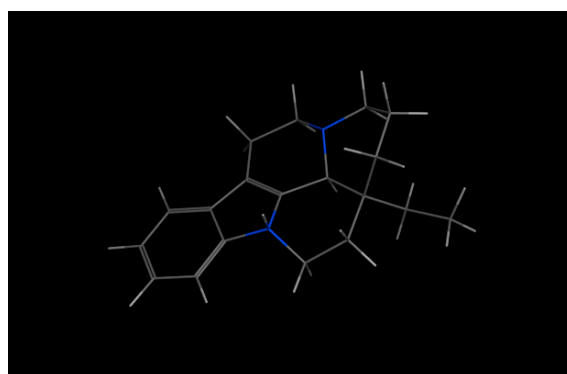
(–)-Eburnamonine (or vincamone)   **Vincane**

Ibogaine

Figure 19: Global minimum conformers of vinca alkaloids and Ibogaine in polytube rendering
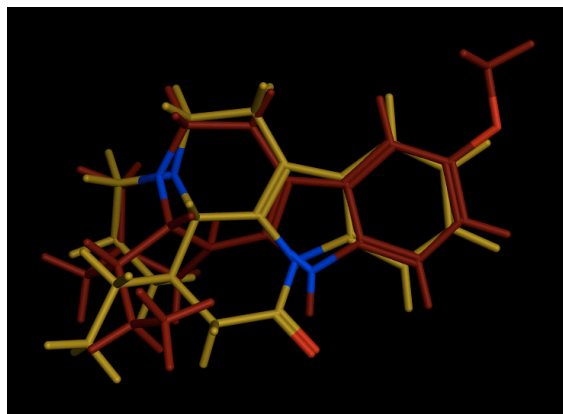
Vicamone (yellow labelled) upon **ibogaine** (red labelled)
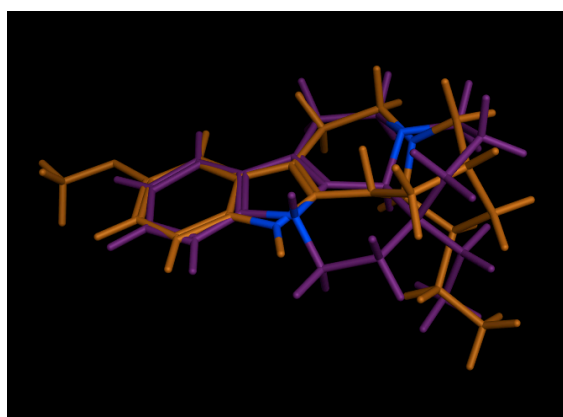
Vincane (purple labelled) upon **ibogaine** (orange labeled)
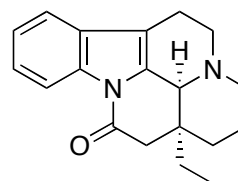
Figure 20: Superimpositions of Vinca structures with ibogaine structure

Designed ligands

In consideration to the information discussed, I have chosen vincane and vincamone (Fig. 21) as lead compounds of two new series of vinca-derivatives, in order to obtain ligands with potential sigma-2 receptor affinity.
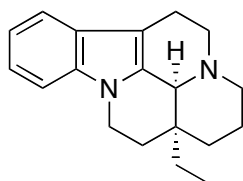
Vincane   **(–)-Eburnamonine** (or vincamone)

Figure 21: lead compounds

For this purpose, an evaluation study was carried out on the chemical accessibility towards appropriate substitutions at the indole moiety. The main issue has been to obtain regioselectivity in aromatic substitution reactions, and to complete the synthetic strategy through the development of Palladiu catalyzed cross-coupling reactions, which have never been described on this scaffold. The achieved target compounds were designed in order to be assessed through binding assays on sigma-1 and sigma-2 receptors. Moreover, the obtained molecules can provide useful information about the use of vinca-scaffold as substrate in cross coupling reactions.

In order to investigate the bulk of the hydrophobic aromatic site in the sigma-2 receptor a phenyl group was added at the indole moiety of vincane. For this purpose, 11-phenyl vincane **(7)** and 10-phenyl vincane **(8)** (Fig. 22) have been designed. The same modification has been made on vincamone structure designing 11-phenyl vincamone (**13**) and 9-phenyl vincamone (**21**) (Fig. 22). The positions were chosen in this case depending

on the regiochemistry of the substrate. The phenyl group was functionalized with chlorine, in order to evaluate its electronic effect on the aromatic properties, leading to compound 11-(4-chlorophenyl) vincamone (**14**) (Fig 22). The achievement of the phenyl vinca-derivatives has permitted the evaluation of Suzuki cross-coupling reactions on this scaffold.[169]

Instead, 11-ethynyl vincamone (**16**) (Fig. 22) was designed to evaluate the Sonogashira[170] cross coupling reaction on vincamone scaffold. Moreover, the presence of an ethynyl group at 11 position of vincamone could give us new information about its influence on the binding properties.

Also the obtained intermediates (Fig. 23) could be assessed in binding studies for sigma receptors. The presence of nitro, amino and iodo substituents at 9 or 11 position of vincamone could provide important information about their electronic effects on the aromatic ring. Vincanol (**2**) and vincamenine (**3**) (Fig. 23) could be useful to evaluate the effects of modifications on the ring termed E (Fig. 17) in vincane scaffold.
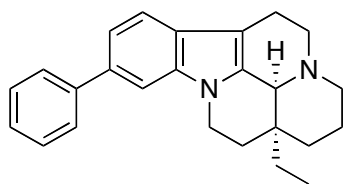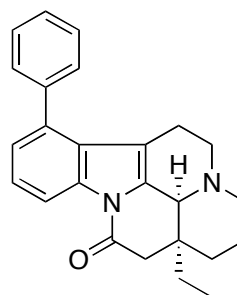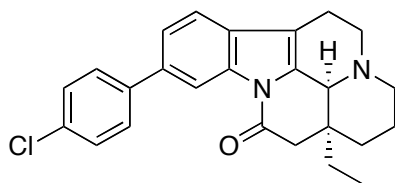
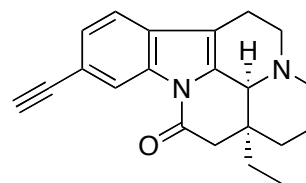11-phenyl vincane **(7)**
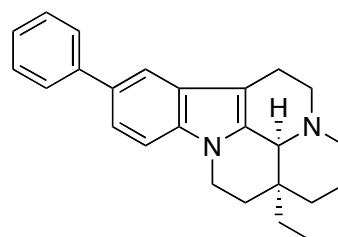
10-phenylvincane (**8**)
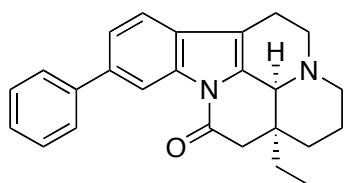
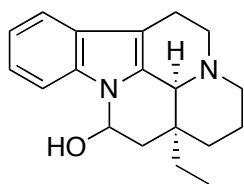11-phenyl vincamone (**13**)

9-phenyl vincamone (**21**)
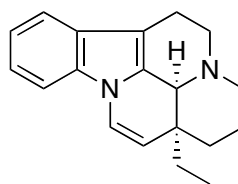
11-(4-chlorophenyl) vincamone (**14**)

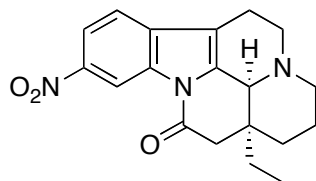11-ethynyl vincamone (**16**)
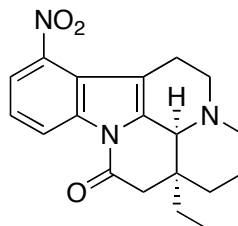
Figure 22: new vinca-derivatives
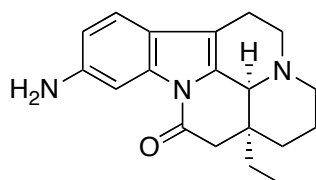
Vincanol (**2**)
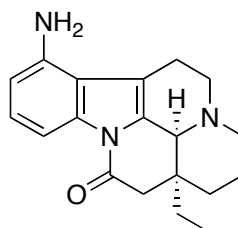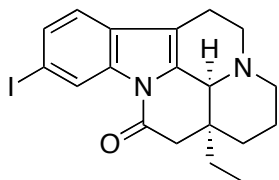

Vincamenine (**3**)
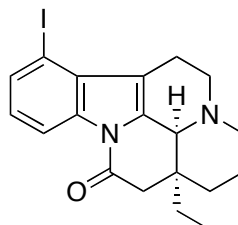

11-nitro vincamone (**9**)

9-nitro vincamone (**16**)

11-amino vincamone (**10**)

9-amino vincamone (**18**)

11-iodo vincamone (**12**)

9-iodo vincamone (**20**)

Figure 23: synthetized intermediates

Chemistry

Both vincane derivatives and vincamone derivatives were obtained using (–)-Eburnamonine (commercially available) as starting compound.

The synthetic pathway of vincane series is illustrated in scheme-1. The (–)-Eburnamonine (**1**) was reduced with LiAlH$_4$ (dust 95%), in anhydrous THF, at 0 °C, under argon atmosphere, over one hour and a half, yield 99,4%.[171] The obtained racemic mixture of vincanol (**2**) was not resolved and was used for the subsequent reductive-dehydration in formic acid 85% at reflux, over two hours.[168] The major product was the vincamenine (**3**), yield 40,65% and the minor product was the vincane (**4**), yield 4,91%. Alternatively, vincamenine (**3**) was prepared from vincanol (**2**) with p-toluensulfonic acid, in anhydrous THF, at reflux over 4 hours, yield 64,2%.[172] The reduction of vincamenine (**3**) to vincane (**4**) was performed in absolute ETOH, using 10% Pd on charcoal, as catalyst, under H$_2$ atmosphere over night, yield 82,6%. Vincane (**4**) was brominated by dropping elemental bromine in a suspension of vincamenine (**3**) and AlCl$_3$ in CHCl$_3$, at 0 °C over half an hour.[173] The bromination of vincane (**4**) resulted in a mixture of three isomers including 9-bromo vincane, 10-bromo vincane (**5**) and 11-bromo (**6**) vincane. Unfortunately, I was only able to isolate 9-bromo vincane (yield 19%) while the other two isomers remained in mixture (yield of the isomeric mixture 38.84%). However the unseparated 10-bromo vincane (**5**) and 11-bromo vincane (**6**) were used as substrates in the subsequent cross coupling reaction, hoping that the two target compounds (**7, 8**) would be isolated. The cross coupling was carried out via Suzuki reaction,[169] with phenylboronic acid, as substituent, tetrakis-Pd(PPh$_3$)$_4$, as catalyst, 2N solution of Na$_2$CO$_3$, as base, in toluene at reflux, under argon atmosphere, over one hour.[169] Unfortunately, also the isolation of the regio-isomers **7** and **8** from their mixture was not achieved (yield of the mixture 41,91%).

The synthetic pathway of the 11-substituted vincamone derivatives is illustrated in scheme 2. Synthesis started with the nitration of indolic moiety obtaining the 11-nitro vincamone (**9**) (yield 60 %) and 9-nitro vicamone (**17**), as side product (yield 23%). The 9-nitro vicamone was used in the preparation of 9-phenyl vincamone (**21**) (scheme 4). The nitration was carried out in a mixture of fuming nitric acid and glacial acetic acid, at 27 °C over two hours.[174] The subsequent reduction, with 5% Pd on charcoal, 10 bar H_2, in absolute ETOH, over an hour and half, led to 11-amino vincamone (**10**) with 90% of yield.[174] The obtained amine (**10**) was processed, via diazonium salt (**11**), with $NaNO_2$/HCl, at temperature -5 °C and then 0-10 °C over 1h. The iodo-derivative (**12**) was obtained adding an aqueous solution of potassium iodide to the diazonium salt (**11**) reaction mixture, at 10-15 °C overnight, yield 33,9%. Suzuki and Sonogashira cross coupling reactions were then performed to achieve the target compounds (**13-14**). Specifically, 11-phenyl vincamone (**13**) was prepared with phenylboronic acid, tetrakis-$Pd(PPh_3)_4$, 2N Na_2CO_3 aqueous solution, toluene at reflux, over 3 hours under Argon atmosphere, yield 37,82%.[169] 11-(4-chlorophenyl) vincamone (**14**) was obtained with 4-Chlorophenylboronic acid, tetrakis-$Pd(PPh_3)_4$, 2N Na_2CO_3 aqueous solution, toluene at reflux, over 3 hours under argon atmosphere, yield 32,17%.[169]

The synthetic pathway of the 11-ethynyl vincamone (**16**) is illustrated in scheme 3. 11-iodio vincamone (**12**) was processed using trimethylsilylacetylene, $Pd(II)(PPh_3)_2Cl_2$, CuI, Isopropylamine, at 35 °C, overnight under argon atmosphere, yielding 77,5% of the trimethylsilylethynyl derivative (**15**).[175] The subsequent desilylation with KF in THF, at room temperature, overnight produced 11-ethynyl vincamone (**16**) with 73,06% of yield.[175]

Scheme 4 illustrates the synthesis of 9-phenyl vincamone (**21**). Synthesis started from 9-nitro vincamone (**17**) was reduced with 5% Pd/Charcoal, in H_2 atmosphere, in

MeOH, at room temperature, over two hours, yielding 94,5% of 9-amino vincamone (**18**).[174] The obtained amine (**18**) was processed, via diazonium salt (**19**), with NaNO$_2$/HCl, at temperature -5 °C and then 0-10 °C over one hour. The 9-iodo vincamone (**20**) was achieved adding an aqueous solution of potassium iodide to the diazonium salt (**19**) reaction mixture, at 10-15 °C overnight, yield 34,76%. Finally, 9-iodo vincamone (**20**) was the substrate of Suzuki reaction, which was carried out with phenylboronic acid, tetrakis-Pd(PPh$_3$)$_4$, 2N Na$_2$CO$_3$ aqueous solution, toluene at reflux, overnight, under argon atmosphere, yielding 72,7% of 9-phenyl vincamone (**21**).[169]

Scheme 1[a]
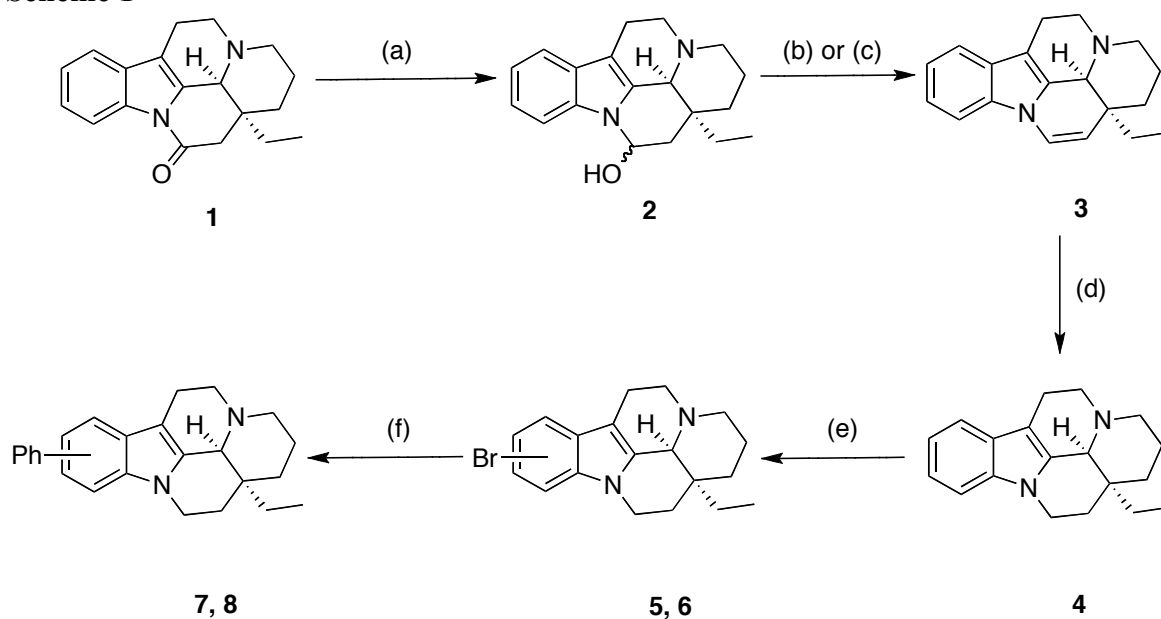
[a]Reagents and conditions: (a) LiAlH$_4$, anhydrous THF, 0 °C, 1.5 h, Ar-atm, (99.4%); (b) HCOOH 85%, reflux, 2h, (40,65%). (c) TsOH, anhydrous THF, reflux, 4h (64,2%); (d) 10% Pd/charcoal, H$_2$-atm, absolute ETOH, 12 h, (82,6%); (e) AlCl$_3$, Br$_2$, CHCl$_3$, 0 °C, 0.5 h, (56,8%); (f) PhB(OH)$_2$, Pd(PPh$_3$)$_4$, Na$_2$CO$_3$, toluene/H$_2$O, at reflux, 1 h, Ar-atm, (41,91%).

Scheme 2[a]
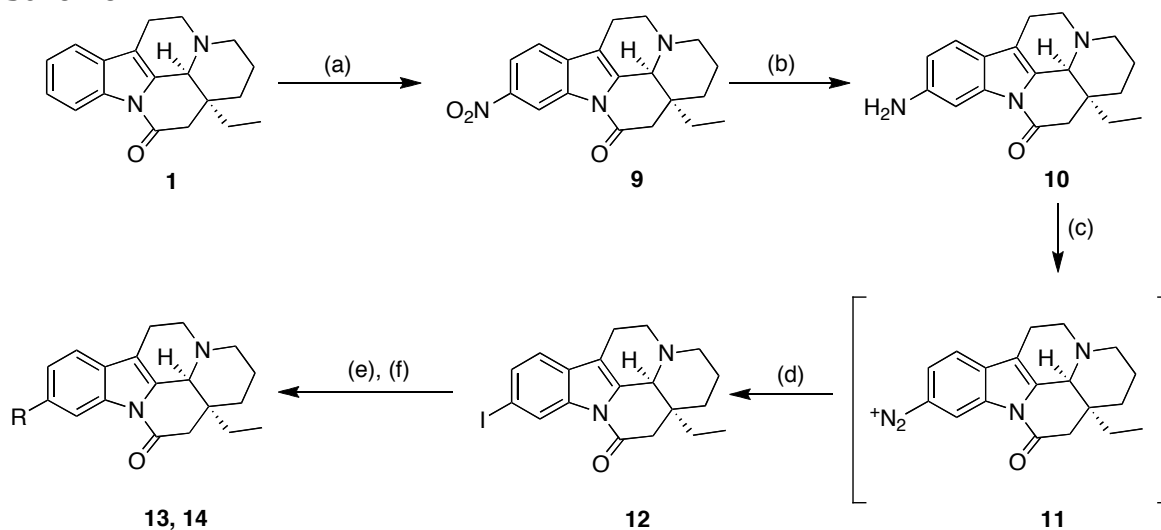
13 R= Phenyl
14 R= 4-chlorophenyl

[a]Reagents and conditions: (a) HNO_3/CH_3COOH mixture, at 27 °C, 2 h, (60 %); (b) 5% Pd/charcoal, 10 bar H_2, absolute ETOH, 1.5 h, (90%); (c) $NaNO_2/HCl$, t between -5 °C and -10 °C, then 0-10 °C over 1h; (d) KI/H_2O, 10-15 °C, 5h then rt overnight, (33,9 %); (e) $PhB(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3, toluene/H_2O at reflux, 3 h, Ar-atm, (37,82%); (f) 4-Chlorophenylboronic acid, Na_2CO_3, toluene/H_2O at reflux, 3 h, Ar-atm, (32,17%).

Scheme 3[a]
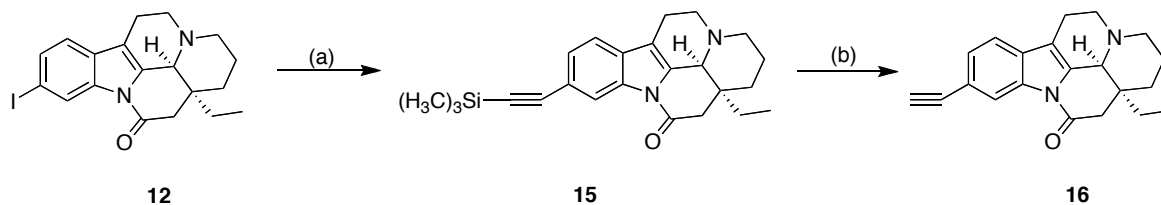
[a]Reagents and conditions: (a) $Pd(II)(PPh_3)_2Cl_2$, CuI, Isopropylamine, 35 °C, Ar-atm, overnight, (77,5%); (b) KF in THF, at rt, overnight, (73,06%).

Scheme 4[a]

17   (a) →   18   (b) →   19   (c) →   20   (d) →   21
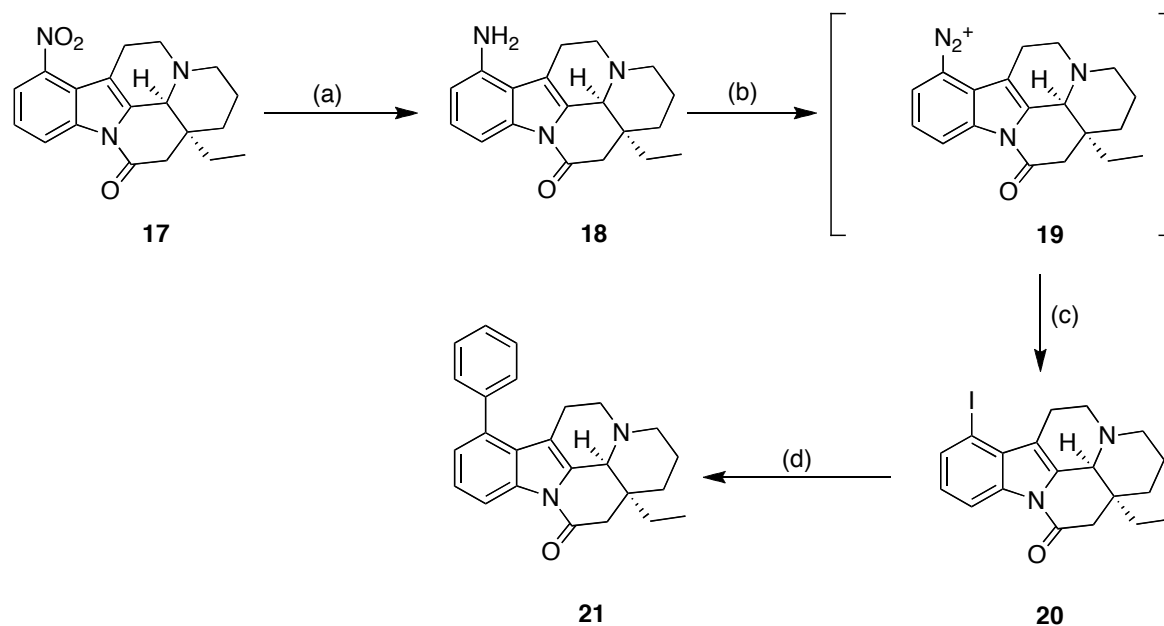
[a]Reagents and conditions: (a) 5% Pd/charcoal, H₂-atm, MeOH, at rt, 2 h, (94,5%); (b) NaNO₂/HCl, t between -5 °C and -10 °C, then 0-10 °C over 1h; (c) KI/H₂O, 10-15 °C, 5h then rt overnight, (34,76%.); (d) PhB(OH)₂, Pd(PPh₃)₄, Na₂CO₃, toluene/H₂O at reflux, overnight, Ar-atm, (72,7%).

Results

As discussed above, substitution reactions on vincane resulted in low yield (19% and 38.84%) and scarce regioselectivity, obtaining three different bromo-derivatives. Moreover, the separation of the obtained regioisomers **5**, **6** was not possible. Instead, vincamone, used as starting material in the nitration reaction, gave quantitative yield and higher regioselectivity. Indeed, only two isomers were obtained and easily isolated through crystallization and flash chromatography, 11-nitro vincamone (**10**) (60% of final yield) and 9-nitrovincamone (**18**) (23%), which were employed to gather two different series of final products. Although, the iodination of vincamone occurred with relatively low yield (34%), it has permitted the obtainment of a key intermediate for the Pd catalyzed cross-coupling reactions. In fact, it has been widely demonstrated that iodine is the best leaving group in Suzuki reactions.[169] The achievement of target compounds **7**, **8**, **13**, **14**, **16** and **21** via Suzuki and Sonogashira reactions demonstrates that Pd chemistry is a useful synthetical strategy in obtaining new vinca derivatives. Hence these results enrich the chemical knowledge of vinca alkaloids and provide useful information for future developments.

Regarding the pharmacological evaluation of the sythetized compound, binding assays at sigma receptors using vincamone, vincane, target compounds and intermediates are still in progress. At the moment only some preliminary data on sigma-1 receptors is available. The binding assay was carried out by labeling sigma-1 receptors with the sigma-1 selective probe, [³H](+)-pentazocine, in guinea pig brain membranes.§

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§ The guinea pig brain membranes were prepared using the procedure described by Matsumoto et al. (Eur. J. Pharmacol. 1995, 280, 301–310). Binding assays were performed as described by DeHaven and co-workers (Eur. J. Pharmacol. 1992, 227, 371–378). Briefly, membrane aliquots (500 mg protein) were incubated with 3 nM [³H]-(+)-pentazocine (29 Ci/mM; K_d = 14 nM) in 50 mM Tris-HCl (pH 7.4). Test compounds were added at concentrations ranging from 10^{-5} to 10^{-11} M. Nonspecific binding was assessed by the addition of 10 mM of haloperidol. The reaction was performed for 150 min at 37 °C and terminated by filtering the solution through Whatman GF/B glass fiber filters that were presoaked for 1 h in a 0.5% poly(ethylenimine) solution. Filters were washed twice with 4 mL of ice-cold buffer. The radioactivity was counted in 4 ml of "Ultima

Vincamone, vincane and 11-phenyl vincamone (**13**) have shown a Ki higher than 1000 nM (table 7). So far these results are in agreement with my research purposes because the assessed compounds show scarce affinity for sigma-1 sites. However, I need data from a more extensive binding assay at sigma-2 sites to verify the predicted sigma-2 affinity and sigma-2 versus sigma-1 selectivity profile of the obtained vinca derivatives.

Table 7. Binding affinities of vinca alkaloids at sigma-1 receptor

Compounds	$K_i \, \sigma_1 \, nM^a$
Vincamone	> 1000
Vincane	> 1000
11-phenyl vincamone (**13**)	> 1000

Values are means ± SEM of three experiments.
^{a}Displacement of 3 nM [3H]-(+)-pentazocine in guinea pig brain membranes.

Final remarks

The development of selective sigma-2 ligands has been the target of considerable research efforts. In this study different vinca-based derivatives were designed and synthesized as potential sigma-2 ligands, aiming to improve the knowledge about sigma-2 receptors and to develop useful drugs for different therapies. Indeed, sigma-2 agonist could be employed in the cancer treatment, whereas sigma-2 antagonist could be used to avoid the motor disorders of the typical anti-psychotics drugs. Moreover, the availability of new selective sigma-2 ligands could encourage the use of sigma-2 receptors as biomarkers in tumor diagnosis.


Gold MV" in a 1414 Winspectral PerkinElmer Wallac liquid scintillation counter. Inhibition constants (*Ki* values) for the tested compounds were calculated using the EBDALIGAND program purchased from Elsevier/Biosoft.

Experimental section

Materials and methods

(-)-Eburnamonine was purchased from Sigma Aldrich Co. or Acros organics. Reagents were purchased from Sigma-Aldrich Co. and Alpha Aesar and used as received. Melting points were determined on Büchi B-540 capillary apparatus and Stuart melting point SMP30 and are uncorrected. Analytical thin-layer chromatography (TLC) was performed on pre-coated silica gel 60 F254 aluminum sheets (Merck); visualization was accomplished under UV or in an iodine chamber. Merck silica gel 60, 230±400 mesh, was used for flash column chromatography. Flash chromatography was also carried out on Flash Master II Argonaut[®] apparatus and on Interchim puriFlash™ 430 evo apparatus. ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian Unity INOVA (200MHz, 400MHz, 500 MHz and 600MHz) spectrometers with TMS as an internal standard, $CDCl_3$ as solvent, δ in ppm, J in Hz. Optical rotations were determined in pyridine (cell = 1dm) (c = 0.4 and 0.2) with ADP220 polarimeter, BS Bellingham+Stanley Ltd. Elemental analyses were measured on an elemental analyzer VarioEL V5.16.5 CHNS mode. Analyses are indicated by symbols of the elements. Molecular weights of some obtained products were determined by fast atom bombardment ionization on a VG-ZAB-2SEQ spectrometer. Analytical HPLC was carried out on a JASCO LC-Net II/ADC apparatus with PU-2080 PLUS pump, UV-2075 PLUS detector, Jasco ChromPass Software Version 1.8 using CHIRALCELL OJ-H 10um 25 x 0.46 chiral column, flow 1ml/min, λ = 254 nm.

Vincanol (2)
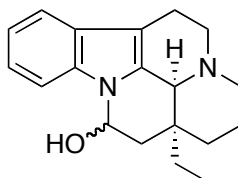
(-)-Eburnamonine (**1**) (5 g, 0.01698 mol, 1 eq), dissolved in anhydrous THF (60 ml), was slowly added by syringe and at 0 ºC to a suspension of $LiAlH_4$ (dust 95%) (1.29 g, 0.03396mol, 2 eq) in anhydrous THF (100 ml). The mixture was stirred for 90 minutes at room temperature. Then cold water (20 ml) was carefully added to the mixture, the obtained suspension was diluted with dichloromethane (200 ml) and filtered under vacuum. The filtrate was dried over Na_2SO_4, filtered again and evaporated under vacuum yielding 5.39g of a white solid. The product was recrystallized in ether yielding 2.885 g of white crystals (**2**). The mother liquors were subsequently dried under vacuum and purified with chromatographic glass column (eluent 95% $CHCl_3$, 5% MeOH in gradient conditions). A total of 4.921 g vincanol (**2**) white solid was obtained. The product was a mixture of two anomers (at 16 position), bi-dimensional NMR spectra (gHSQC e gHMBC with 400MHz apparatus) demonstrated that major component (71% according to the ^{1}HNMR integrals ratio) was the anomer with the hydroxyl group in axial configuration (assigned chemical shift values in Fig. 24). The anomeric mixture of vincanol was not resolved and used as substrate in the subsequent step.

Yield of anomeric mixture (**2**) 97.7 %. $C_{19}H_{22}N_2O$; M.W. 296.19; TLC R_f 0,51 (9/1

CH$_2$Cl$_2$/MeOH); mp 93-96 ° (decomposition).

^{1}H NMR chemical shift values (ppm scale)
ax = axial protons; eq = equatorial protons

^{13}C NMR chemical shift values (ppm scale)
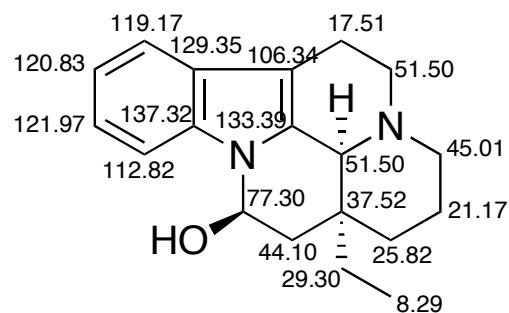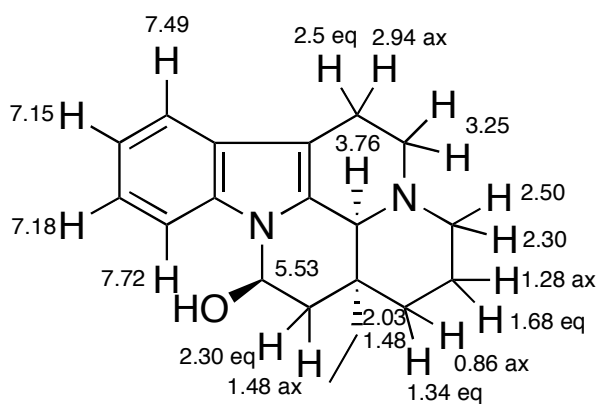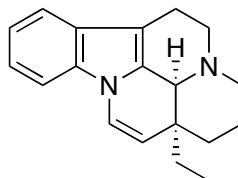
Figure 24: assigned ^{13}C and ^{1}H chemical shift values for vincanol (axial anomer), measured with Varian 400MHz spectrometer
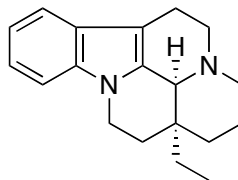
Vincamenine (3)

Procedure A - Vincanol (**2**) (1.807 g, 6.0965 mmol) in 85% formic acid (9.83 g) was stirred over 2 hours at reflux conditions. The excess of formic acid was partially evaporated under vacuum and the residue was poured into ice water (100ml). The PH-value of the obtained solution was adjusted with an aqueous 1N sodium hydroxide aqueous solution to 8-9 and the precipitation of sodium formate was observed. The aqueous phase was extracted five times with 120 ml of dichloromethane. The organic phase was dried over anhydrous magnesium sulphate, filtered and evaporated under vacuum, obtaining a yellow oily product. Purification was carried out with flash chromatographic apparatus (eluent $CHCl_3$ / MeOH 9.5 / 0.5 in gradient conditions) obtaining 690 mg of vincamenine (**3**) (yield 40.65%) and 0.84 g of vincane (**4**) (yield 4.91%), both products were pale yellow oils.

Procedure B - Vincanol (536 mg, 1.8084 mmol) was dissolved in anhydrous toluene (7ml) and p-toluensulphonic acid (0.5 mg, 0.002815 mmol) was added to the solution. The mixture was refluxed over 4 hours. Then the toluene was evaporated under vacuum and the yellow residue was taken up in 30 ml of boiling ethyl acetate. The resultant solution was filtered and evaporated under vacuum. Purification was made with flash chromatography (eluent 95% $CHCl_3$ / 5% MeOH in gradient conditions) obtaining 0.323 g of Vincamenine (**3**) yellow oil.

Yield 64.2 %; $C_{19}H_{22}N_2$; M.W. 278.39 g/mol; TLC R_f 0.23 (95% $CHCl_3$, 5% MeOH). ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.47 (d, 1H, $J = 7.8$), 7.33 (d, 1H, $J = 7.8$), 7.19-7.17 (m, 1H), 7.12-7.09 (m, 1H), 6.92 (d, 1H, $J = 8.4$), 5.08 (d, 1H, $J = 8.4$), 4.27 (s, 1H), 3.39-3.35 (m, 1H), 3.30-3.25 (m, 1H), 3.07-3.00 (m, 1H), 2.77-2.72 (m, 1H), 2.69-2.67 (d, 1H) 2.53-2.50 (m, 1H), 2.02-1.95 (m, 1H), 1.77-1.68 (m, 2H), 1.49-1.41 (m, 2H), 1.18-1.13 (m, 1H), 0.99 (t, 3H, $J = 7.8$). ^{13}C NMR (400MHz, $CDCl_3$) δ 134.29, 130.83, 128.92, 122.27, 120.58, 120.47, 119.11, 117.41, 109.21, 107.76, 56.57, 52.85, 46.15, 38.04, 31.78, 28.27, 21.49, 17.21, 9.69.
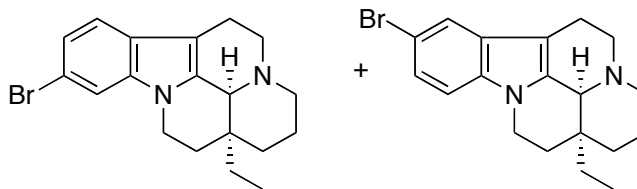
Vincane (4)

Vincamenine (**3**)(0.69g, 2.47 mmol) was dissolved in absolute ethanol (4 ml) and added to a pre-hydrogenated suspension of 10% palladium on charcoal (0.124 g) in absolute ethanol (4ml). The reaction mixture was kept overnight under hydrogen atmosphere and vigorous stirring. The catalyst was filtered off and the filtrate was evaporated to dryness. Flash chromatography eluent 20/1 = CH_2Cl_2/MeOH afforded 574 mg of pale yellow oil (**4**). Then the product was crystallized in ether obtaining white crystals.

Yield 82.6%; $C_{19}H_{24}N_2$; M.W. 280.41 g/mol; mp 81.5-84.5 ºC (from ether); TLC R_f 0.16 (95% $CHCl_3$, 5% MeOH); elemental analysis calc. for $C_{19}H_{24}N_2$: C 81.38%, N 9.99%, H 8.63%; elemental analysis found: C 81.02%, N 9.89%, H 8.62%; HPLC R.T. 15.80 (n-Hexane / i-Propanol 95 / 5), purity grade 99.92%; ^1H NMR (600 MHz, $CDCl_3$) δ 7.48 (d, 1H, J = 7.8Hz), 7.28 (d, 1H, J = 8.4), 7.17-714 (m, 1H) 7.11-7.09 (m, 1H), 4.157-4.125(m, 1H), 3.89 (s, 1H), 3.79-3.74 (m, 1H), 3.33-3.24 (m, 2H), 3.01-2.95(m, 1H), 2.59-2.55 (m, 2H), 2.47-2.42 (m, 1H), 2.16-2.10 (m, 1H), 1.99-1.90 (m, 2H), 1.81-1.74 (m, 1H), 1.58-1.52 (m, 1H), 1.38-1.35 (m, 1H), 1.30-1.25 (m, 1H), 1.10-1.05 (m, 1H), 0.93 (t, 3H, J = 7.8). ^{13}C NMR (600MHz, $CDCl_3$) δ 136.95, 128.65, 121.15, 119.94, 118.76, 109.88, 104.98, 59.88, 51.90, 45.25, 39.08, 34.73, 32.55, 29.60, 24.49, 21.41, 17.76, 8.22.
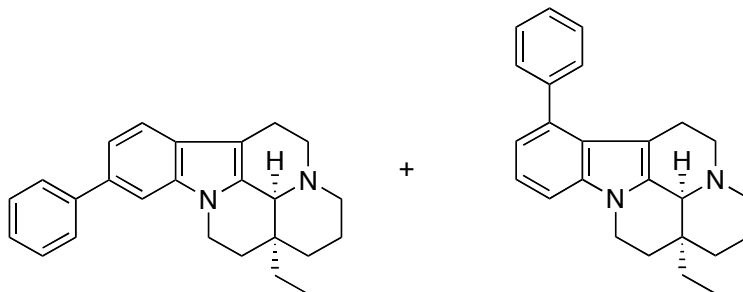
10-bromo vincane and 11-bromo vincane (5, 6)

Vincane (**4**) (0.677 g, 2.41mmol) and AlCl$_3$ (anhydrous powder 99.99 %) (0.322 g, 2.41mmol) were suspended at 0 °C in CHCl$_3$ (15 ml). Elemental bromine (0. 4239 g, 2.65 mmol, 0,13 ml) was added dropwise to the suspension under stirring at 0 °C. After completion of the addition the mixture was stirred at 0 ºC for half an hour. The mixture was alkalinized with 2N ammonia solution. The rusty brown precipitate was filtered off, and the two phases of the filtrate were separated. The water phase was extracted three times with dichloromethane. The combined organic phases were washed with water, dried with magnesium sulphate, filtered and evaporated under vacuum. The product was lyophilized and gave a yellow foam weighing 764mg. The Flash chromatography with eluent ethyl acetate has produced 0.155 g of 9-bromo vincane and 0.337 g of a mixture consisting of 10-bromo vincane and 11-bromo vincane (**5, 6**) (yellow oil). The major component of the mixture was the 11-bromovincano (72% according to ^{1}HNMR integrals ratio).

Yield of isomeric mixture (**5, 6**) 38.84%; C$_{19}$H$_{23}$BrN$_2$; M.W. 359.30 g/mol; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.59 (d, 1H), 7.45 (d, 1H), 7.31 (d, 1H), 7.24-7.11 (m, 3H), 4.16-4.03 (m, 2H), 3.83 (s, 2H), 3.80-3.70 (m, 2H), 3.36-3.19 (m, 4H), 2.98-2.83 (m, 2H), 2.61-2.29 (m, 6H), 2.19-2.04 (m, 3H), 1.99-1.72 (m, 6H), 1.59-1.47 (m, 2H), 1.41-1.23 (m, 6H), 1.07-0.97 (m, 2H), 0.91 (m, 6H).

10-phenyl vincane and 11-phenyl vincane (7, 8)

The isomeric mixture of 10-bromo vincane and 11-bromo vincane (**5, 6**) (0.308 g, 0.8572 mmol, 1eq) was dissolved in 1,2-dimethoxyethane (4ml). Phenylboronic acid (0.125 g, 1.02864 mmol, 1.2 eq) and 2N solution of Na_2CO_3 (0,85 ml) was added to the bromo vincane solution. Argon was flushed over 10 minutes into the reaction vessel, then the catalyst tetrakis-Pd(PPh$_3$)$_4$ (0.0495g, 0.04286 mmol, 5%) was added and the mixture was refluxed under argon over 1 hour. After the end of the reaction the mixture was filtered over Celite® 535 and evaporated under vacuum. The residue was taken up with dichloromethane (40ml) and washed 3 times with water. The organic phase was dried over anhydrous magnesium sulphate, filtered and dried under vacuum, obtaining a semi-solid weighing 0.362 g. The purification was carried out with flash chromatographic apparatus (eluent: ethyl acetate), obtaining 0.128 g of yellow oil (mixture of isomers **7, 8**). The product was crystallized in n-Hexane.

Yield: 41.91% (mixture of isomers); $C_{25}H_{28}N_2$; 356.50 g/mol; mp 126.5-130.5 (decomposition), 140-141.5; ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.67 (d, 2H, J = 6.8), 7.54 (d, 1H, J =8), 7.48-7.42 (m, 2H), 7.38-7.35 (m, 1H), 7.33-7.29 (m, 1H), 7.20-7.17 (m, 1H), 4.22-4.18 (m, 1H), 3.91 (s, 1H), 3.85-3.78 (m, 1H), 3.36-3.24 (m, 2H), 3.04-2.96 (m, 1H),

2.61-2.57 (m, 2H), 2.49-2.42 (m, 1H), 2.19-2.05 (m, 1H), 1.98-1.90 (m, 2H), 1.83-1.73 (m, 1H), 1.60-1.51 (m, 1H), 1.39-1.25 (m, 3H), 1.13-1.07 (m, 1H), 0.99 (m, 3H). ^{13}C NMR (400MHz, CDCl$_3$) δ 143.70, 143.41, 137.49, 134.81, 134.37, 131.09, 131.02, 129.34, 128.36, 128.30, 128.08, 127.07, 126.50, 126.34, 120.74, 119.83, 118.96, 111.79, 110.10, 108.54, 105.00, 59.93, 51.84, 45.28, 39.15, 34.76, 32.55, 30.39, 29.59, 24.54, 21.44, 17.79, 8.25.

11-nitrovincamone (9) and 9-nitrovincamone (16)
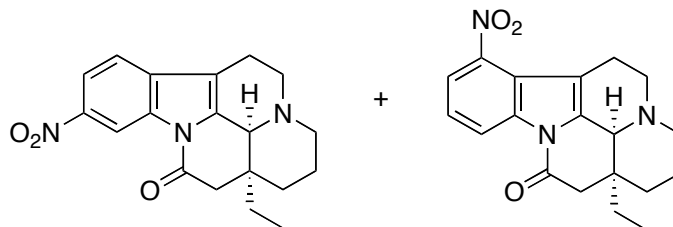
(-) Eburnamonine (**1**) (0.500 g, 1.698 mmol) was dissolved in glacial acetic acid (6ml) and the obtained solution was added dropwise to a mixture of fuming nitric acid (100%) (2.40 ml, 3.66g, d = 1.52 g/ml) and glacial acetic acid (99-100 %) (2.40 ml, 2.52g, d = 1.05 g/ml). After two hours of stirring at 27° C, the mixture was poured in ice water (100ml) and the PH value was adjusted with 25% aqueous ammonia solution to 8-9. The aqueous suspension was extracted 3 x 60 ml with dichloromethane. The combined organic phases were dried over magnesium sulphate, filtered and evaporated under vacuum, obtaining a yellow solid weighing 620mg. The product was recrystallized from methanol obtaining yellow crystals (0.226 g) consistent in the isomer 11-nitrovincamone (**9**). The mother liquors were concentrated at rotavapor and purified in flash chromatography apparatus, with eluent ethyl acetate, obtaining the isomer 9-nitro vincamone (**16**) yellow solid (0.133 g) and the remaining 11-nitrovincamone yellow solid (0.120 g) (**9**).

11-nitro vincamone (**9**):

yield 60%; $C_{19}H_{21}N_3O_3$; M.W. 339.39; TLC R_f 0.12 (EtOAc); mp 196.5-197.5 (methanol); $[\alpha]_{20}^{D}$ = -125° (pyridine, c = 0.4); elemental analysis calc. for $C_{19}H_{21}N_3O_3$: C 67.24%, N 12.38%, H 6.24%; elemental analysis found: C 66.14%, N 12.16%, H 6.09%; ^{1}H NMR (600 MHz, CDCl$_3$) δ 9.15 (s, 1H), 8.14 (dd, 1H, J = 8.4 Hz, 1.2 Hz), 7.44 (d, 1H, J = 8.4

Hz), 4.02 (s, 1H), 3.37-3.34 (m, 1H), 3.29-3.26 (m, 1H), 2.91 (s, 1H), 2.74-2.62 (m, 3H), 252-2.48 (m,1H), 2.37-2.33 (m, 1H), 2.08-2.04 (m, 1H), 1.78-1.68 (m, 2H), 1.54 (d, 1H, J = 13.8 Hz), 1.41 (d, 1H, J = 13.8 Hz), 1.01-0.93 (m, 4H); ^{13}C NMR (600MHz, CDCl$_3$) δ 168.15, 145.34, 138.88, 135.55, 133.56, 120.02, 118.47, 113.32, 112.93, 58.43, 51.06, 45.22, 44.80, 39.45, 28.99, 27.86, 21.19, 16.91, 8.30.

9-nitro vincamone (**16**):

yield 23%; C$_{19}$H$_{21}$N$_3$O$_3$; M.W. 339.39; TLC R$_f$ 0.41 (EtOAc); mp 131.5-134; HPLC R.T. 24.36 (n-Hexane / i-Propanol 70 / 30), purity grade 99,84%; elemental analysis calc. for C$_{19}$H$_{21}$N$_3$O$_3$: C 67.24%, N 12.38%, H 6.24%; elemental analysis found: C 65.74%, N 12.08%, H 6.24%; $[α]_{20}^D$ = +10° (pyridine, c = 0.2); ^{1}H NMR (600 MHz, CDCl$_3$) δ 8.75 (d, 1H, J = 8.4 Hz), 7.99 (d, 1H, J = 7.8 Hz), 7.34 (dd, 1H, J = 8.4Hz, 7.8 Hz), 4.02 (s, 1H), 3.33-3.29 (m, 1H), 3.22-3.17 (m, 1H), 3.13-3.09 (m, 1H), 2.80-2.78 (m, 1H), 2.71 (d, 1H, J = 16.8 Hz), 2.64 (d, 2H, 16.8 Hz), 2.44-2.40 (m, 1H), 2.08-2.05 (m, 1H), 1.79-1.75 (m, 1H), 1.69-1.66 (m, 1H), 1.52 (d, 1H, J = 13.8 Hz), 1.41 (d, 1H, J = 13.8 Hz), 1.03-0.98 (m, 1H), 0.93 (t, 3H, J = 7.8 Hz); ^{13}C NMR (600MHz, CDCl$_3$) δ 168.64, 142.56, 137.22, 136.92, 124.16, 124.14, 122.37, 121.37, 112.15, 58.87, 51.66, 45.10, 44.93, 39.17, 29.01, 27.80, 21.17, 20.23, 8.27.
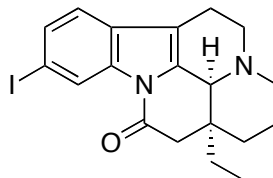
11-amino vincamone (10)
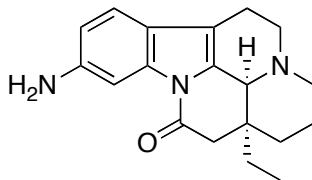
11-nitro vincamone (**9**) (0.500 g, 1.4733 mmol) was dissolved in absolute ethanol (240 ml) and the solution was divided in three vessels (80ml solution per each vessel), then 5% Pd/Charcoal (0.50 g per each vessel) was added. Each vessel was located in autoclave, with 10 bar maximum pressure of hydrogen, at room temperature, over 90 minutes. The three reaction mixtures were filtered, on paper filter, and the combined filtrates were dried under vacuum, obtaining a white foamy solid (0.463 g) (**10**). The product was recrystallized in methanol obtaining 0.298 g of yellow crystals (**10**)(purity 99.18 %, according to HPLC 70/30: n-hexane/iso-propanol) and the dried mother liquors produced a foamy solid weighing 0.111 g (**10**) (purity 96,83% according to HPLC 70/30: n-hexane/iso-propanol).

Yield 89.7%; $C_{19}H_{23}N_3O$; 309.41 g/mol; TLC R_f 0.37 (CH_2Cl_2:MeOH 95:5); mp 223.5-227.5 °C (methanol). HPLC R.T. 29.18 (n-hexane / i-propanol 70 / 30); ^{1}H NMR (400 MHz, CDCl$_3$) δ 7.76 (d, 1H, J = 2.4 Hz), 7.18 (d, 1H, J = 8 Hz), 6.66 (dd, 1H, J = 8 Hz, 2.4 Hz), 3.93 (s, 1H), 3.74 (br, 2H), 3.33-3.28 (m, 1H), 3.25-3.17 (m, 1H), 2.88-2.79 (m, 1H), 2.64-2.52 (m, 3H), 2.45-2.39 (m, 2H), 2.09-1.99 (m, 1H), 1.80-1.58 (m, 2H), 1.47 (d, 1H, J = 13.6 Hz), 1.40-1.37 (m, 1H), 1.06-0.98 (m, 1H), 0.91 (t, 3H, J = 7.6 Hz); ^{13}C NMR (400MHz, CDCl$_3$) δ 168.36, 144.99, 136.22, 129.93, 123.09, 119.27, 113.31, 113.14, 103.67, 58.39, 51.39, 44.97, 39.01, 38.85, 29.02, 27.54, 21.28, 17.33, 8.32.

11-iodo vincamone (12)

11-amino vincamone (**10**) (0.450g, 1.454 mmol, 1 eq) was suspended in concentrate hydrochloric acid (1.75ml) at temperatures between -10 and -5 °C. At the same temperatures a solution of $NaNO_2$ (0.118 g, 1.718 mmol, 1.2 eq in 0.5 ml of water) was slowly added by syringe under the surface of the suspension. After completion of the addition, the solid material was completely dissolved (yellow solution). The mixture was stirred over 1 hour at temperature between -5 and 0 °C. Then a solution of potassium iodide (1.20g, 7.27 mmol, 5eq in 2.65 ml of water) was slowly added (in one hour) to the reaction mixture under vigorous stirring at 10-15 °C, forming a rusty brown precipitate. The reaction mixture was stirred overnight at room temperature. Then water was added to the mixture and the PH value adjusted to 8-9 with 2N ammonia solution. The aqueous solution was extracted with dichloromethane (3 x 100 ml) and the combined organic phases were washed with brine (3 x 30ml) and then with sodium thiosulphate (2 x 25ml), The colour of the organic phase changed from red to yellow. The organic phase was dried over magnesium sulphate, filtered and evaporated to dryness, yielding an orange-yellow solid (0.398 g). Flash chromatography eluent ethyl acetate afforded 0.266 g of an orange solid (**12**). The solid product was triturated with n-hexane and ethanol yielding a yellow solid (**12**) (0.207 g).

Yield 33.9 %; $C_{19}H_{21}IN_2O$; M. W. 420.29; TLC R_f 0.50 (CH_2Cl_2 : MeOH 95 : 5); mp 237-239 (n-hexane); HPLC R.T. 7.53, purity grade 97.06% (n-hexane / i-propanol 60 / 40); elemental analysis calc. for $C_{19}H_{21}IN_2O$: C 54.30%, N 6.67%, H 5.04%; elemental analysis found: C 54.62%, N 6.68%, H 5.06%; MS: m/z 421.2 [M]+; ^{1}H NMR (600 MHz, CDCl$_3$) δ 8.74 (s, 1H), 7.57 (d, 1H, J = 7.8 Hz), 7.16 (d, 1H, J = 7.8 Hz), 3.95 (s, 1H), 3.34-3.31 (m, 1H), 3.26-3.21 (m, 1H), 2.89-2.83 (m, 1H), 2.67-2.57 (m, 3H), 2.46-2.33 (m, 2H), 2.06-2.02 (m, 1H), 1.76-1.64 (m, 2H), 1.49 (d, 1H, J = 13.8 Hz), 1.39 (d, 1H, J = 13.8 Hz), 1.01-0.91 (m, 4H).

11-phenyl vincamone (13)
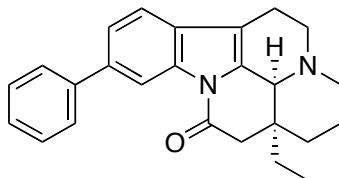
Phenylboronic acid (0.139 g, 1.142 mmol, 1.2 eq) and 2N solution of Na_2CO_3 (0.95 ml, 2eq) were added to a solution of 11-iodio vincamone (**12**) (0.400 g, 0, 9517 mmol, 1eq) in toluene (38 ml). Argon was flushed over 10 minutes into the reaction vessel, then the catalyst tetrakis-Pd(PPh$_3$)$_4$ (0,055 g, 0,0475 mmol, 5%) was added. The mixture was refluxed under argon over two and a half hours. After the reaction ended the mixture was diluted with water (50ml) and extracted with dichloromethane (3 x 50ml). The combined organic phases were dried over sodium sulphate, filtered and evaporated to dryness under vacuum, yielding brown oil (0.490g). The purification was carried out with flash chromatographic apparatus using ethyl acetate/methanol (95% / 5%) eluent and obtaining a yellow oil weighing 0.180 g (**13**). The product was triturated in n-Hexane and ethanol, obtaining 0.070g of a yellow solid (**13**) (purity grade 97.6% determined with HPLC 50% / 50% n-hexane: iso-propanol).

Yield 39.7%, $C_{25}H_{26}N_2O$, M.W. 370.49; TLC R$_f$ 0.24 (ethyl acetate : methanol 95:5); mp 191-193 (n-hexane/methanol); HPLC R.T. 14.09 (n-hexane : i-propanol 50% : 50%); MS: m/z 371.1 [M]+; ^{1}H NMR (600 MHz, CDCl$_3$) δ 8.65 (d, 1H, J = 1.2 Hz), 7.68 (m, 2H), 7.55 (dd, 1H, J = 8.4 Hz, 1.2 Hz), 7.49-7.44 (m, 3H), 7.34 (t, 1H, J = 7.2 Hz), 4.04 (s, 1H), 3.40-3.37 (m, 1H), 3.30-3.28 (m, 1H), 2.94-2.93 (m, 1H), 2.71-2.60 (m, 3H), 2.56-2.53 (m, 1H), 2.47-2.44 (m,1H), 2.10-2.06 (m, 1H), 1.81-1.79 (m, 1H), 1.70-1.66 (m, 1H), 1.524 (d,

1H, J = 13.2 Hz), 1.42 (d, 1H, J = 13.2 Hz), 1.09-1.07 (m, 1H), 0.94 (t, 3H, J = 7.8 Hz);

^{13}C NMR (600MHz, CDCl$_3$) δ 166.19, 142.19, 138.69, 135.63, 129.80, 129.41, 128.13, 127.69, 124.00, 118.97, 115.59, 113.11, 58.51, 51.37, 45.08, 44.96, 39.16, 29.07, 27.54, 21.16, 17.21, 8.33.
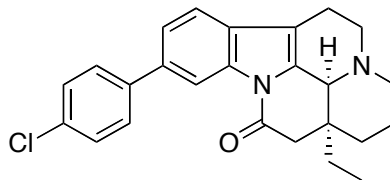
11-(4-chlorophenyl) vincamone (14)

4-chlorophenylboronic acid (0.134 g, 0.8565 mmol, 1.2 eq) and 2N solution of Na_2CO_3 (0.71 ml, 2eq) were added to a solution of 11-iodio vincamone (**12**) (0.300 g, 0, 7138 mmol, 1eq) in toluene (20 ml). Argon was flushed over 10 minutes into the reaction vessel, then the catalyst tetrakis-Pd(PPh$_3$)$_4$ (0,041 g, 0,0357 mmol, 5%) was added. The mixture was refluxed under argon over 3 hours. After the reaction ended the mixture was diluted with water (30ml) and extracted with dichloromethane (5 x 30ml). The combined organic phases were dried over sodium sulphate, filtered and evaporated to dryness under vacuum, obtaining a brown solid (0.390 g). The purification was carried out with flash chromatographic apparatus using ethyl acetate, as eluent, obtaining 0.045 g of yellow solid (**14**).

Yield 16%; $C_{25}H_{25}ClN_2O$; M.W. 404.93 g / mol; TLC R$_f$ 0.21 (ethyl acetate); ^{1}H NMR (500 MHz, CDCl$_3$) δ 8.60 (s, 1H), 7.59 (m, 2H), 7.48 (m, 2H), 7.40 (m, 2H), 3.99 (s, 1H), 3.364-3.312 (m, 1H), 3.29-3.22 (m, 1H), 2.96-2.88 (m, 1H), 2.67-2.56 (m, 3H), 2.52-2.47 (m, 1H), 2.44-2.389 (m, 1H), 2.09-2.02 (m, 1H), 1.81-1.72 (m, 1H), 1.70-1.62 (m, 1H), 1.52-1.47 (m, 1H), 1.41-1.38 (m, 1H), 1.07-1.01 (m, 1H), 0.93 (t, 3H, J = 8 Hz); ^{13}C NMR (500MHz, CDCl$_3$) δ 167.68, 139.99, 136.50, 134.87, 133.03, 132.86, 132.07, 129.50, 128.83, 128.61, 128.48, 123.00, 118.38, 114.69, 112.45, 57.76, 50.62, 44.40, 44.33, 38.44, 28.34, 26.99, 20.60, 16.54, 7.63.

11-trimethylsilylethynyl vincamone (15)
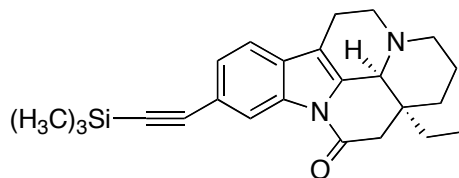
CuI (0.0063g, 0.0333 mmol, 4%) and Pd(II)(PPh3)$_2$Cl$_2$ (0.0291 g, 0.04164 mmol, 5%) were added to a suspension of 11-iodo vincamone (**12**) (0.350 g, 0,832 mmol, 1eq) in fresh distilled isopropylamine (100 ml). The mixture was stirred 15 minutes under argon atmosphere then trimethylsilylacetilene (0.090g, 0.13 ml, 0.916 mmol, 1.1 eq) was added to the suspension. The reaction was stirred at 35 °C, overnight, under argon atmosphere. The reaction mixture was filtered and the filtrate was evaporated under vacuum. Flash chromatography eluent ethyl acetate afforded 0.252 g of white solid (**15**).

Yield 77.5 %; C$_{24}$H$_{30}$N$_2$OSi; M.W. 390.59 g/mol; TLC R$_f$ 0.28 (ethyl acetate); HPLC R.T. 4.14 (n-hexane / iso-propanol 60% / 40%); ^{1}H NMR (600 MHz, CDCl$_3$) δ 8.46 (s,1H), 7.33 (d, 1H, J = 8.4 Hz), 7.11 (d, 1H, J = 8.4 Hz), 4.07 (s, 1H), 3.38-3.35 (m, 1H), 3.30-2.23 (m, 1H), 2.90-2.83 (m, 1H), 2.70-2.58 (m, 3H), 2.55-2.51 (m, 1H), 2.45-2.38 (m, 1H), 2.08-2.03 (m, 2H), 1.86-1.76 (m, 1H), 1.62-1.55 (m, 1H), 1.53-1.41 (m, 2H), 1.15-0.98 (m, 1H), 0.93 (t, 3H, J = 7.8 Hz), 0.26 (s, 9H).

11-ethynyl vincamone (16)
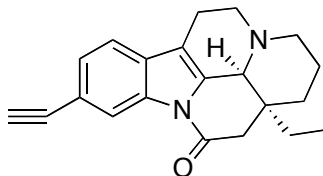
Potassium fluoride (0.068g, 1.1778 mmol) was added to a suspension of 11-trimethylsilylethynyl vincamone **(15)** (0.230 g, 0.5889 mmol) in THF (20ml) and the mixture was stirred at room temperature overnight. The mixture was filtered on silica gel and the filtrate was evaporated to dryness. Flash chromatography with eluent chloroform yielded 0.137 g of a white solid (**16**).

Yield 73.06%; $C_{21}H_{22}N_2O$; M.W. 318.41; TLC R_f 0.31 (ethyl acetate); mp 201.5-205 (decomposition)(from $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.53 (s, 1H), 7.41 (d, 1H, J = 8.4 Hz), 3.46 (d, 1H, J = 8 Hz), 4.03 (s, 1H), 3.39-3.25 (m, 1H), 3.10 (s, 1H), 2.93-2.86 (m, 1H), 2.71-2.60 (m, 1H), 2.53-2.39 (m, 3H), 2.13-2.04 (m, 1H), 1.85-1.66 (m, 2H), 1.52 (d, 1H, 13.6), 1.42 (d, 1H, 13.6), 1.27 (s, 1H), 1.06-0.94 (m, 4H); ^{13}C NMR (500MHz, $CDCl_3$) δ 167.36, 133.71, 133.23, 130.22, 127.87, 120.03, 117.97, 117.77, 112.54, 84.40, 76.67, 57.68, 50.56, 44.41, 44.42, 38.53, 28.33, 26.88, 20.38, 16.34, 7.61.

9-amino vincamone (18)
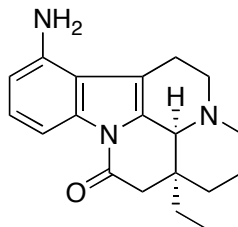
9-nitro vincamone (**17**) (2.55 g, 7.513 mmol) was partially dissolved in methanol (200 ml) and added to a prehydrogenated suspension of 5% Pd/charcoal (0.255 g, 0.1 fold in weight). The mixture was stirred at room temperature under hydrogen atmosphere over 2 hours. The reaction mixture was filtered over Celite® 545 and the filtrate was evaporated to dryness, obtaining a white solid (2.197 g) (**18**).

Yield 94.5%; $C_{19}H_{23}N_3O$; 309.41 g/mol; TLC R_f 0.39 (dichloromethane/methanol 9.5/0.5); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.78 (d, 1H, J = 7.6 Hz), 7.19-6.98 (m, 1H), 6.46 (d, 1H, J = 7.4 Hz), 3.85 (m, 3H), 3.30-3.05 (m, 3H), 2.62-2.31 (m, 5H), 2.02-1.92 (m, 1H), 1.71-1.19 (m, 4H), 1.02-0.82 (m, 4H).

9-iodo vincamone (20)
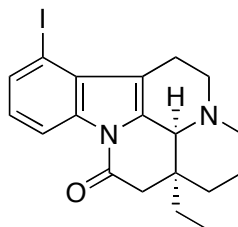
9-amino vincamone (**18**) (2.1 g, 0.0068 mol, 1 eq) was suspended in concentrate hydrochloric acid (10 ml) at temperatures between -10 and -5 °C. At the same temperature a solution of $NaNO_2$ (0.558 g, 0.0081 mol, 1.2 eq, in 2 ml of water) was slowly added by syringe under the surface of the suspension. After completion of the addition, the solid material was completely dissolved (yellow solution). The mixture was stirred over one hour at temperatures between -5 and 0 °C. Then a solution of potassium iodide (5.62g, 0.0339 mol, 5eq in 12.4 ml of water) was slowly added to the reaction mixture under vigorous stirring at 10-15 °C, forming a rusty brown precipitate. The reaction mixture was stirred overnight at room temperature. Then water was added to the mixture and the PH value adjusted to 8-9 with 2N ammonia solution. The aqueous solution was extracted with dichloromethane (3 x 150 ml) and the combined organic phases were washed first with brine (2 x 100ml) and then with sodium thiosulphate (2 x 50 ml). The organic phase was dried over magnesium sulphate, filtered and evaporated to dryness, yielding a red solid (2.79 g). Flash chromatography eluent cyclohexane/ethyl acetate 8/2 afforded 0.99 g of a yellow solid (**20**).

Yield 34.76 %; $C_{19}H_{21}IN_2O$; MW 420.29 g/mol; TLC R_f 0.22 (cyclohexane/ethyl acetate 8/2); mp 198.3-200 °C; ^1H NMR (200 MHz, $CDCl_3$) δ 8.41 (d, 1H, J = 8.2 Hz), 7.67 (d,

1H, J = 7.8 Hz), 7.02-6.94 (m, 1H), 3.89 (s, 1H), 3.36-2.85 (m, 4H), 2.69-2.36 (m, 4H), 2.14-1.95 (m, 1H), 1.85-1.36 (m, 4H), 1.07-0.88 (m, 4H); ^{13}C NMR (200 MHz, CDCl$_3$) δ 167.49, 134.86, 134.54, 133.15, 131,93, 125,49, 115.92, 113.72, 83.83, 57.81, 50.59, 44.30, 44.15, 38.21, 28.30, 26.89, 20.52, 19.11, 7.58.

9-phenyl vincamone (21)
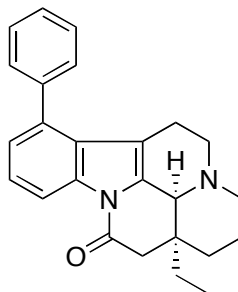
9-iodio vincamone (**20**) (0.300 g, 0, 7138 mmol, 1eq) was dissolved in toluene (7 ml), phenylboronic acid (0.104 g, 0.8565 mmol, 1.2 eq) and 2N solution of Na_2CO_3 (0.71 ml, 2eq) were added to the solution. Argon was flushed over 10 minutes into the reaction vessel and then the catalyst tetrakis-Pd(PPh$_3$)$_4$ (0,041 g, 0,0357 mmol, 5%) was added. The mixture was refluxed under argon overnight. After the reaction ended the mixture was diluted with water (30ml) and extracted with dichloromethane (3 x 50ml). The combined organic phases were dried over sodium sulphate, filtered and evaporated to dryness under vacuum, obtaining a yellow oily product (0.331g). The purification was carried out with flash chromatography using eluent cyclohexane/ethyl acetate 8/2, obtaining 0.192 g of pale yellow oil (**21**).

Yield 72.7 %; $C_{25}H_{26}N_2O$; M.W. 370.49; TLC R$_f$ 0.21 (cyclohexane/ethyl acetate 8/2); ^{1}H NMR (500 MHz, CDCl$_3$) δ 8.452 (d, 1H, J = 8.0 Hz), 7.43-7.38 (m, 5H), 7.33 (dd, 1H, J = 7.5 Hz, 8 Hz), 7.164 (d, 1H, J = 7.5 Hz), 3.96 (s, 1H), 3.11-3.02 (m, 2H), 2.64 (dd, 2H, J = 16.5 Hz, 36.5 Hz), 2.50 (m, 1H), 2.40-2.33 (m, 2H), 2.07-1,99 (m, 1H), 1.95-1.90 (m, 1H), 1.78-1.69 (m, 1H), 1.67-1.60 (m, 1H), 1.50 (d, 1H, J = 14), 1.40-1.19 (m, 2H), 1.11-1.05 (m, 1H), 0.92 (t, 3H, J = 7 Hz). ^{13}C NMR (500 MHz, CDCl$_3$) δ 167.74, 140.56, 135.13,

134.41, 132.20, 129.62, 127.75, 127.58, 127.15, 125.31, 123.87, 115.21, 112.70, 57.91, 50.85, 44.32, 38.26, 28.32, 26.93, 20.58, 19.17, 7.59.

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