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"Either write something worth reading or do something worth writing."

Benjamin Franklin

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When I decided to develop maturity in myself, I aimed to make the world a better place for living. In my opinion, scientists were the right people to afford this responsibility. Therefore, I decided to enter scientific research world.

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Abstract

Positron emission tomography (PET) is a nuclear medicine imaging technique and a modern non-invasive diagnosis tool for different diseases. In PET, a biologically active molecule which can target a function, metabolic process or endogenous macromolecule and which is tagged by a positron-emitting isotope, is tracked *in vivo*. In this work, we aimed to develop a novel targeting molecule for visualization of beta-2 adrenergic receptors in human body with PET. In the first phase of the work, we identified a lead structure of beta-2 adrenergic ligands from a literature search and designed a group of potent analogues of the structure. Six compounds, the racemic mixtures of *(1-(3-((9H-carbazol-4-yl)oxy)-2-hydroxypropyl)piperidin-4-yl)(4-fluorophenyl)methanone* (VIEBAR11), *N-(1-(3-((9H-carbazol-4-yl)oxy)-2-hydroxypropyl)piperidin-4-yl)-4-fluorobenzamide* (VIEBAR52), *N-(1-(3-((9H-carbazol-4-yl)oxy)-2-hydroxypropyl)piperidin-4-yl)-4-methoxybenzamide* (VIEBAR53) and their (*S*)-enantiomers were synthesized in decent yields. Receptor binding affinity (K_i) towards the target protein (β_2) as well as β_2/β_1 and β_2/β_3 selectivity ratios were determined using a competitive radioligand binding assay. All compounds displayed high affinity and good selectivity for the beta-2 adrenergic receptor as K_i values were in the low nanomolar range (0.3 nM – 1.4 nM). Hence, radiolabeling of the respective candidates and further preclinical evaluations can be conducted in the near future.

Zusammenfassung

Die Positronen-Emissions-Tomographie (PET) ist eine nuklearmedizinische Bildgebungstechnik und ein modernes, nicht invasives Diagnosewerkzeug für verschiedene Krankheiten. In der PET wird ein biologisch aktives Molekül, das auf eine Funktion, einen Stoffwechselvorgang oder ein endogenes Makromolekül zielen kann, durch ein Positronen emittierendes Isotop markiert und *in vivo* verfolgt.

In dieser Arbeit zielten wir darauf ab, einen neuartigen Liganden für die Visualisierung von beta-2-Adrenozeptoren im menschlichen Körper mit PET zu entwickeln. In der ersten Phase der Arbeit identifizierten wir eine Leitstruktur von beta-2-adrenergen Liganden aus einer Literatursuche und entwarfen eine Gruppe von potentiellen Analoga der Struktur. Sechs Verbindungen, VIEBAR11, VIEBAR52, VIEBAR53 und deren S-Enantiomere wurden in zufriedenstellenden Ausbeuten synthetisiert. Rezeptorbindungsaffinität (K_i) und β_2/β_1 und β_2/β_3 Selektivitätsverhältnisse wurden unter Verwendung eines Radioligandenbindungssays bestimmt. Die K_i -Werte waren in einem geeigneten Bereich (niedrig nanomolar) für alle Verbindungen. Daher werden nun in nächster Zukunft Markierungen mit PET-Nukliden und weitere präklinische Experimente durchgeführt.

Chapter I:

Introduction

1.1. Aim

The most important prerequisite for precise treatment of a disease, is the diagnosis. There are various types of medical diagnostic techniques used for identification of disorders and diseases. Among those, molecular imaging techniques are the most important for non-invasive medical diagnosis (Weissleder & Mahmood 2001). Nowadays, molecular imaging is not only used for the purpose of diagnosis, but also applied in the process of therapy (e.g. primary staging, therapy control, re-staging).

Typical molecular imaging methods are optical imaging, near infrared imaging, magnetic resonance imaging (MRI), single photon emission computer tomography (SPECT) and positron emission tomography (PET). One of the main applications of these techniques is the investigation of the molecular pathways involved in diseases such as cancer, cardiovascular and neurological disorders and diseases (Jadvar & Parker 2006; Kitson et al. 2009). We intended to target and track a group of neurological functions in central nervous system (CNS) as well as peripheral nervous system (PNS), by visualization of beta-2 adrenergic receptors with PET.

Adrenergic receptors, which belong to a class of receptors known as G-protein coupled receptors, are the molecular targets of catecholamines (Minneman et al. 1981). Endogenous catecholamines, such as epinephrine and norepinephrine are able to activate both major types of adrenergic receptors, alpha-adrenoceptors (α -AR) and beta-adrenoceptors (β -AR) (Small et al. 2003).

In order to visualize β 2-ARs via PET in human body, selective ligands are required. However, selective β 2-AR ligands for PET are limited. [^{11}C]formoterol and [^{11}C]procaterol, the selective β 2-AR agonists, and [^{11}C]ICI 118,551, a selective β 2-AR blocker, demonstrated insignificant biodistribution in the CNS tissues in animal samples (Visser et al. 1998; Visser et al. 2000; Moresco et al. 2000). More recently, Stephenson et al. introduced a series of beta blockers, which demonstrated better uptake into cortex and cerebellum in ex-vivo rodents studies. However, Stephenson's compounds didn't show a favorable cardiac biodistribution (K. A. Stephenson et al. 2008; K. Stephenson et al. 2008).

One of the most important challenges regarding the development of PET-tracers for neuroimaging is the penetration of the blood-brain barrier (BBB). Statistical studies have suggested a

range of values concerning physicochemical properties to influence the BBB permeability (Pike 2009). For example, to cross the BBB a compound should have a polar surface area (PSA) less than 90 \AA^2 , molecular weight (MW) less than 500 g/mol, a Log of partition coefficient (LogP) between 2 and 5, and hydrogen bond donors (HBD) fewer than 3 (Hitchcock & Pennington 2006). Based on these parameters, we aimed to find a comprehensive PET-tracer applicable for both cerebral/cortical and peripheral adrenergic systems. Tasler et al. identified a group of beta adrenergic receptor ligands from which two compounds demonstrated appropriate selectivity for β_2 -adrenoceptors (Tasler et al. 2010). Due to their high receptor binding affinity and selectivity, VIEBAR11 ($K_{i\beta_1}=32 \text{ nM}$, $K_{i\beta_2}=0.3 \text{ nM}$, $K_{i\beta_3}=250 \text{ nM}$), was chosen as the lead structure and its analogues were designed so they should be able to penetrate the BBB (Tasler et al. 2010).

In this thesis, the synthesis, receptor binding affinity and β_2/β_1 and β_2/β_3 selectivity ratios of three β_2 -AR antagonists and respective *S*-enantiomers *VIEBAR11*, *VIEBAR31*, *VIEBAR42*, *VIEBAR43*, *VIEBAR52* and *VIEBAR53* (Kamali Sarvestani et al. 2017) are reported.

1.2. Historical background

First sights for understanding receptors and their functions were obtained in early 20th century. A British physiologist at Cambridge University, John Newport Langley (1852–1925), provided the concept of “receptive substance” in 1905 which criticized hypothesized direct action of several drugs or poisons on cell (Perez 2007; Maehle 2004; Langley 1905). Seemingly 1936 Nobel Prize laureate, Henry Dale, used the receptor concept in relation of nervous system for first time. He claimed the ergot alkaloids prevent motor action of epinephrine and have no influence on its inhibitory action due to a mechanism he called “receptive mechanism of adrenaline” (Dale 1906; Perez 2007; Schild 1997). Back in 1901, John Jacob Abel purified “epinephrin” in Baltimore, United states whereas the Austrian biochemist, Otto von Fürth called it “Suprarenin” (Abel. 1899; Perez 2007; von Fürth 1899). Later on Jokichi Takamine (1854–1922) crystallized epinephrine as the first isolated hormone in 20th century (Yamashima 2003). The American pharmaceutical company Parke, Davis & Co registered the word “adrenalin” for the substance and Henry Dale used “adrenaline” in 1906 (Tansey 1995). The psychobiologist Otto Loewi published several papers about neurochemical transmission in toad and frog in 1920s and showed adrenaline is the sympathetic transmit-

ter in amphibians (Loewi 1922; Ginsborg 1969). In 1936, Loewi and Dale shared the Nobel Prize of Physiology or Medicine for their “discoveries relating to chemical transmission of nerve impulses” (Ginsborg 1969). Mary Lilius Hare described an enzyme in 1928, which nowadays called monoamine oxidase (Hare 1928). A paper published in 1937 by Hermann Blaschko demonstrated that the enzyme can oxidize dopamine, adrenaline and noradrenaline (Blaschko et al. 1937). German scientist, Peter Holtz predicted biochemical reactions from tyrosine to dopamine, noradrenaline and adrenaline at end of the decade 1930 (Holtz 1939). Several years later, von Euler declared that noradrenaline is a sympathetic neurotransmitter (von Euler 1945; von Euler 1946). In 1948, the American pharmacologist Raymond Ahlquist found out that adrenergic receptors behave differently and belong to two classes, that oppose to each other in stimulation and inhibition properties. He called them “alpha adrenotropic receptors” (currently α -adrenergic receptors) and “beta adrenotropic receptors” (currently β -adrenergic receptors) (Ahlquist 1948). In 1950s, secondary messenger systems and specifically cyclic adenosine monophosphate (cAMP) was discovered by the American biochemist, Earl Wilbur Sutherland who was awarded the Nobel Prize of Physiology or Medicine for his attempts to describe “the mechanisms of the action of hormones” (Blumenthal 2012). After a while, James Black et al. developed propranolol as the first clinical antagonist for β -adrenergic receptors (Black et al. 1964). β -adrenoceptors were differentiated into two subtypes β_1 and β_2 in 1967 (Lands et al. 1967). Classification of α -adrenoceptors in two categories, postsynaptic (α_1) and presynaptic (α_2) was brought forward in 1970s (Langer 1974) when radioligand binding assay technique was developed for adrenergic receptors (Goldstein et al. 1971; Alexander et al. 1975; Perez 2007; U’Prichard et al. 1978; Greenberg et al. 1976).

1.3. Sympathetic nervous system

The human nervous system consists of two sections, the central nervous system (CNS) and the peripheral nervous system (PNS). Sophisticated network of nerves, which connect the CNS to other parts of the body form the PNS (Rea 2016). Nervous system comprises of nerve cells, which are called neurons. Neurons communicate with each other through the chemical or electrical signals. The communication site of a neuron is synapse. Neurons out of CNS are found in ganglion (Webb & Adler 2017; Rea 2016). PNS, which is

principally divided to somatic nervous system (SoNS), includes cranial and spinal nerves and autonomic nervous systems (ANS). According to the anatomical and physiological differences ANS is subdivided to sympathetic and parasympathetic systems (Laight 2013; Franklin 2017; Webb & Adler 2017). These both work together and their function is almost antagonistic (Webb & Adler 2017). The sympathetic division is the stimulator of the fight-or-flight response (Rea 2016). Fight-or-flight response is a physiological feedback from an organism in confront with a danger, attack or suddenly movement. Neurons release acetylcholine and norepinephrine to activate acetylcholine and adrenergic receptors.

1.4. Adrenergic receptors

G-Protein coupled receptors

G-protein coupled receptor (GPCR) superfamily consists of the cell membrane receptors which are stimulated by an extracellular molecule out of a cell to transmit a signal along cell membrane. A GPCR has an extracellular amine terminal connected to seven membrane-spanning α -helical segments to which three intra- and three extracellular loops are linked and the molecular chain is ended to an intracellular carboxyl terminal (see Figure 1) (Rosenbaum et al. 2009; Kobilka 2007). GPCRs are classified into five families based on the structural similarities: rhodopsins (Family A), the secretins (Family B), glutamates (Family C), adhesion family and the frizzled/taste family (Rosenbaum et al. 2009; Kobilka 2007; Fredriksson et al. 2003). Functionally, a GPCR is activated by an external signal mediator. G-proteins stimulate and inhibit the enzyme adenylyl-cyclase via their two sub units Gs and Gi, respectively where they activate phospholipase C (PLC) via Gq protein.

Adrenergic receptors

Adrenal medulla, the innermost part of adrenal gland secretes epinephrine (80%) and norepinephrine (20%) in response to stimulation of sympathetic neurons (McCorry 2007). Epinephrine and norepinephrine belong to a group of organic compounds called catecholamines, active amines with a catechol moiety. Catecholamines are capable to stimulate and activate adrenergic receptors. Adrenergic receptors are made up of two main groups (Table 1): α -adrenergic receptors (α -ARs) and β -adrenergic receptors (β -ARs) (Small et al. 2003; Van

Waarde et al. 2004). α -ARs, with two subtypes α_1 and α_2 , are responsible mainly for smooth muscle contraction (Civantos Calzada et al. 2001; Vanhoutte & Miller 1989; Matthews et al. 1984; Muramatsu et al. 1990) and the vasoconstriction of blood vessels (Coffman & Cohen 1988; Langer et al. 1985).

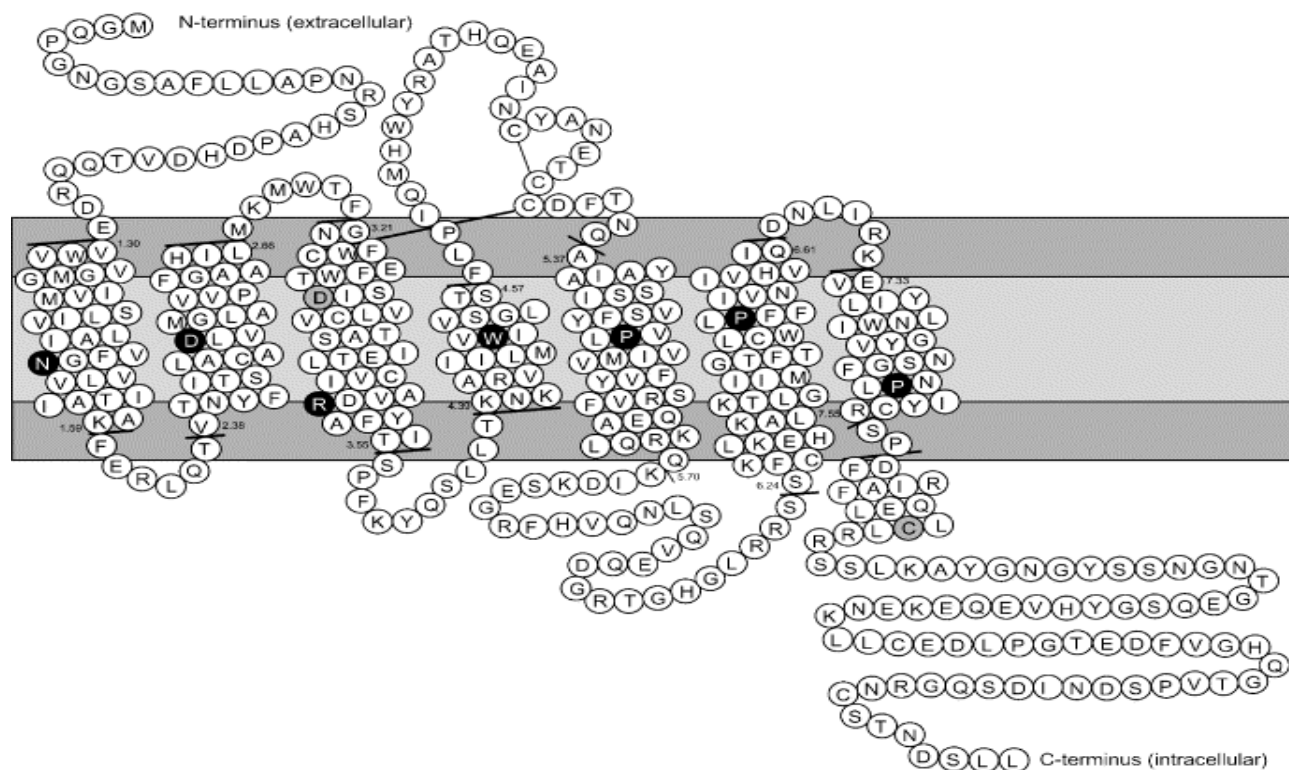


Figure 1. Schematic structure of beta-2 adrenoceptor as a GPCR (Kristiansen 2004)

On the other hand, β -ARs are classified in the subtypes β_1 , β_2 , β_3 and probably β_4 (Sarsero et al. 1999; Minneman et al. 1981). There are three highly homologous subtypes of α_1 -ARs, (α_{1A} , α_{1B} and α_{1D}) which mediate a wide range of physicochemical responses to endogenous and pharmacologically synthesized catecholamines. Previously known α_{1C} -AR subtype is now classified under α_{1A} -AR (Graham 1996; Cotecchia 2010). In all subtypes of α_1 -ARs, a heterotrimeric G protein, Gq, activates phospholipase C to increase Ca^{2+} via voltage-dependent, transient receptor potential channels (TRPC3 or TRPC6) or to release it from the intracellular reservoirs (Cotecchia 2010). Smooth muscle contraction is the main function of α_1 -ARs (Robertson & Biaggioni 2012). In contrast, α_2 -ARs mediate their effects via inhibitory Gi proteins. Based on their pharmacological properties, they are divided into four subtypes: α_{2A} , α_{2B} , α_{2C} and α_{2D} , whereby the α_{2D} subtype is a variant of the human α_{2A} adrenoceptor finding in rodents (Ruuskanen et al. 2004). α_2 -adrenoceptors are present in high

density both in pre- and postsynaptic nerve terminals in CNS and PNS. In the central nervous system, activation of α 2-adrenoceptors leads to predominantly inhibitory effects: inhibition of neurotransmitter release, hypothermia, analgesia, sedation, euphoric effects and central blood pressure reduction. Peripheral functions include vasoconstriction and promotion of platelet aggregation (Giovannitti et al. 2015).

Table 1. Adrenergic receptor subtypes, Location in body and function

AR sub-type	Tissue	Function
α 1	Vascular smooth muscle	Smooth muscle contraction
α 2	Adrenergic nerve terminal	Inhibition of transmitter release
β 1	Heart	Increase heart rate and contraction
β 2	Vascular smooth muscle	Smooth muscle relaxation
β 3	Adipose tissue	Lipolysis, Thermogenesis

β 1-adrenergic receptors which are present in heart can influence the heart rate, the contractility and the excitatory conduction of the heart. These receptors can stimulate releasing of the enzyme renin in the kidneys. In contrast, β 2-ARs are distributed throughout the human body especially in the smooth muscles of the bronchial tubes, the blood vessels, and the uterus. They promote smooth muscle relaxation and activate glycogenolysis. β 2-adrenoceptors are also expressed in lymphatic organs on immune cells and can thus influence the release of different cytokines. β 3-adrenergic receptors are mainly located in the brown adipose tissue and are associated with lipolysis and thermogenesis (Held et al. 2013).

1.5. Beta-2 adrenoceptors

β 2-ARs are membrane-bound receptors coupled to G-proteins, and are present in various tissues with different functions. This receptor is coupled to the $G_{S\alpha}$ subunit of G protein, which activates adenylate cyclase. This enzyme catalyses the formation of cyclic adenosine monophosphate (cAMP), which subsequently activate protein kinase A (Johnson & M 2006; Liggett 1999; Sears & Lötval 2005). Action of the β -ARs is part of the fight-or-flight response

and thus they can stimulate heart muscle contraction, dilatation of blood vessels, glycogenolysis and smooth muscle relaxation. The latter pharmacological response is very important in treatment or control of asthma since it facilitates respiration in bronchi (Morgan et al. 1982; Wu et al. 2015; Johnson & M 2006; Stein et al. 2000).

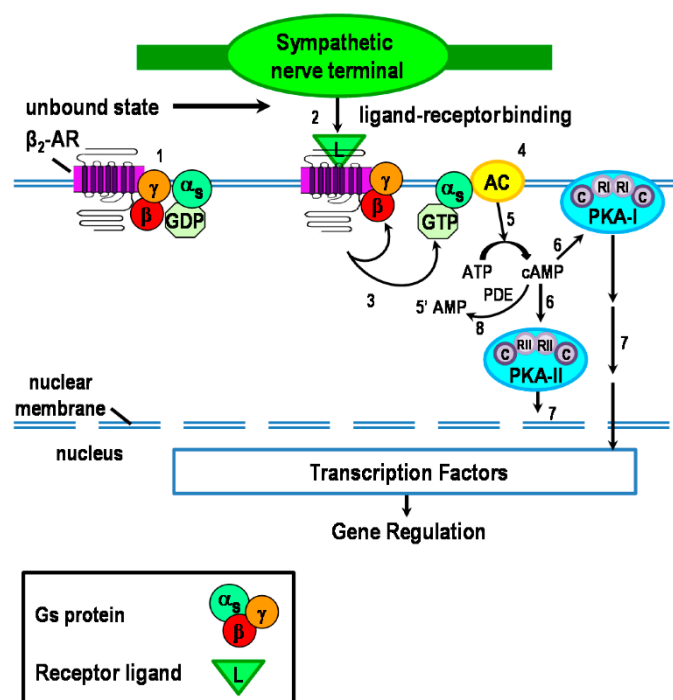


Figure 2. β_2 -adrenergic receptor (AR) signaling pathway (Lorton et al. 2015)

Epinephrine binds to β_2 -adrenoceptors to activate them. Norepinephrine also can activate these receptors with thirty-fold lower affinity than epinephrine (Chung et al. 1988). The binding of these ligands stabilizes the active site of the receptor. In this state, the receptor can activate intracellular G proteins and thus initiating a signal transduction cascade (Figure 2). A signal transduction can be carried out via G protein-dependent and G protein-independent mechanisms. Hence, the binding of agonist-activated GPCRs to the heterotrimeric G proteins facilitates the exchange of guanosine diphosphate for guanosine triphosphate. Then, G protein is dissociated into the subunits $G\alpha$ and $G\beta\gamma$. While $G\beta\gamma$ mainly affects the function of ion channels, $G\alpha$ subunits of Gs proteins interact with membrane-adenylate cyclase and stimulate their function. Therefore a "second messenger" cAMP is formed from ATP. The hydrolysis of GTP by $G\alpha$ causes the association of subunits again to the heterotrimeric G protein, which can bind receptors again (Pedersen & Ross 1982).

1.6. Known ligands

Adrenocorticotrophic hormone (ACTH) and sympathetic nervous system (SNS) stimulate secretion of epinephrine and norepinephrine so that the heart and respiratory rates are increased and the body gets ready to encounter to the special condition (Jansen et al. 1995; Valenta et al. 1986; James and Brown 1997).

Table 2. β_2 -AR ligands, $-\log K_d$ values and selectivity versus β_1 -AR

Ligand	$-\log K_d$	$\beta_2:\beta_1$	Ref.
S-cyanopindolol	11	5	(Baker 2010)
Carazolol	10.5	6.3	(Baker 2010)
SDZ 21009	10.3	22	(Baker 2010)
Bucindolol	10	4.8	(Baker 2010)
Salmeterol	9.3	3388	(Baker 2010)
Pindolol	9.2	4.6	(Baker 2010)
ICI 118,551	9	71	(Moresco et al. 2000; Hoffmann et al. 2004)
Formoterol	8.6	331	(Baker 2010)
Zinterol	8	120	(Baker 2010)
Procaterol	7.1	200	(Baker 2010)
Isoprenaline	6.6	4	(Baker 2010)
Epinephrine	6.2	9.6	(Baker 2010)
Norepinephrine	5.4	0.48	(Baker 2010)

Epinephrine and norepinephrine function in the human body as the hormones and the neurotransmitters. Chemically, both are classified in the family of catecholamines. Norepinephrine is synthesized in adrenal medulla and nerve terminals in central nervous system (CNS) from the reaction of dopamine with dopamine β -monooxygenase; whereas epinephrine is produced from methylation of the primary amine of norepinephrine (Figure 3). In clinical pharmacology, β_2 -adrenoceptor ligands are a crucial target for different types of diseases. Clenbuterol, fenoterol, reproterol, salbutamol, salmeterol and terbutaline, are used for the treatment of bronchial asthma and other respiratory diseases (Sears & Lötvall 2005). Moreover, in obstetrics β_2 -adrenoceptor agonists such as fenoterol, are administered as tocolytics (Engelhardt et al. 1997). In contrast to the agonists of the β_2 -adrenergic receptor, selective antagonists have no noteworthy therapeutic significance. Non-selective beta-blockers which block both β_1 - and β_2 adrenergic receptors (e.g. propranolol) help the treatment of hypertension, heart failure and coronary heart disease application. In-vivo studies have shown several β -

adrenoceptor ligands such as [^{11}C]CGP-12177 and (S)-1- ^{18}F]Carazolol are appropriate for detection of β -adrenoceptors in lung and heart (Moresco et al. 2000). Since all types of adrenoceptors are localized in CNS and peripheral nervous system (PNS), selectivity ratio of the ligands are important.

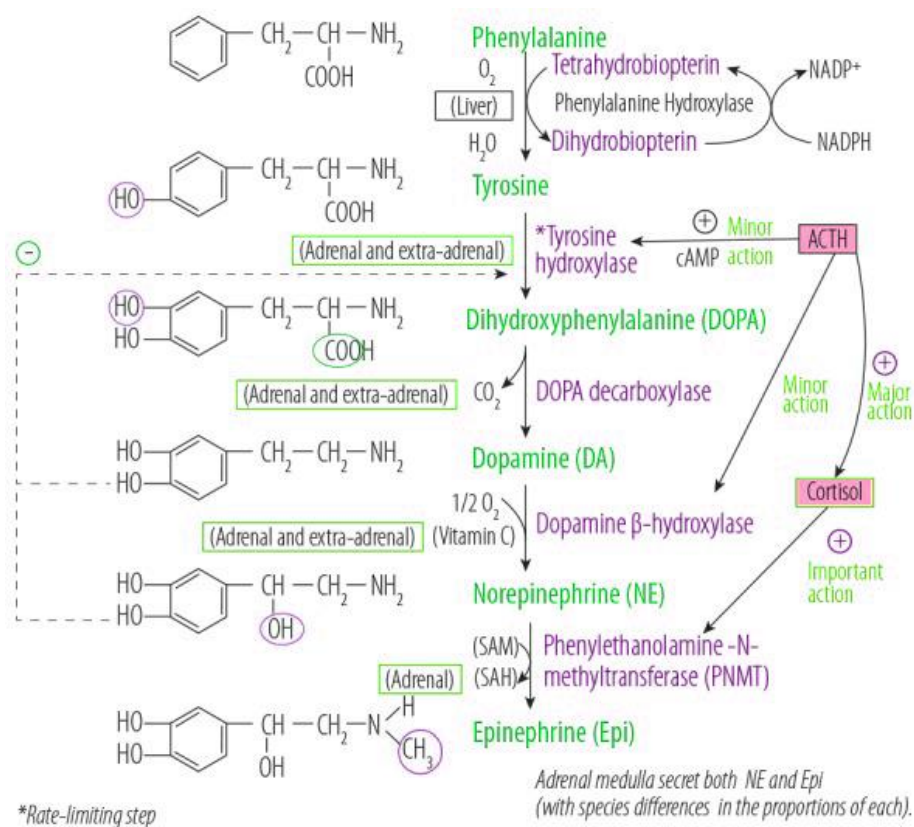


Figure 3. Biosynthesis of epinephrine and norepinephrine. Adapted from (Mula-Abed et al. 2015)

Up to this point, a large amount of catecholamines have been produced as the analogues of epinephrine and norepinephrine (Table 2). Among the selective β_2 -AR ligands, Salbutamol, Salmeterol, Clenbuterol, Terbutaline, Formoterol and Fenoterol are used for treatment of asthma. Nevertheless, ICI 118,551 as an antagonist of β_2 -ARs, has a selectivity ratio 100 for $\beta_2:\beta_1$ (Moresco et al. 2000). On the other hand, Butoxamine, Salmeterol, Procaterol and Formoterol are reported as the selective β_2 ligands. According to the ligand binding affinity values of the β_2 -agonists and antagonists, Salmeterol, Formoterol and ICI 118,551 have a suitable binding affinity and selectivity (Baker 2010; Moresco et al. 2000).

1.7. Beta-2 adrenergic receptors in clinical medicine

β 2-ARs play a significant role in the treatment of respiratory diseases, such as chronic obstructive pulmonary disease (COPD) and asthma, as well as neurodegenerative disorders in central nervous system (CNS) and cardiovascular diseases (Fonarow 2009; Sears & Lötvall 2005; Kalaria et al. 1989; Johnson & M 2006). Smooth muscle relaxation, inhibition of acetylcholine release from cholinergic nerve terminals, stimulation of serous and mucous cell secretion, promotion of water movement into the airway lumen, increase in bronchial blood flow, reduction in venular permeability and inhibition of mediator release from some inflammatory cells are the tasks mediated by β 2-ARs in bronchial tissues (Bai & TonyR. 1992). In CNS, β 2-ARs are expressed by glial cells, particularly astrocytes (Mantyh et al. 1995) and could be addressed as a target for astrocytosis (Sutin & Griffith 1993; Roy & Sontheimer 1995; Griffith & Sutin 1996). Some investigations support a role of β 2-ARs in diagnosis and treatment of prostate cancer (Braadland et al. 2015), heart failure and cardiac disorders (Choi 2005; Wang et al. 2015).

1.8. PET-imaging

Conventional imaging approaches such as Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) are suitable for visualization of anatomic changes; but these methods are not accurate enough for understanding diseases in molecular scale.

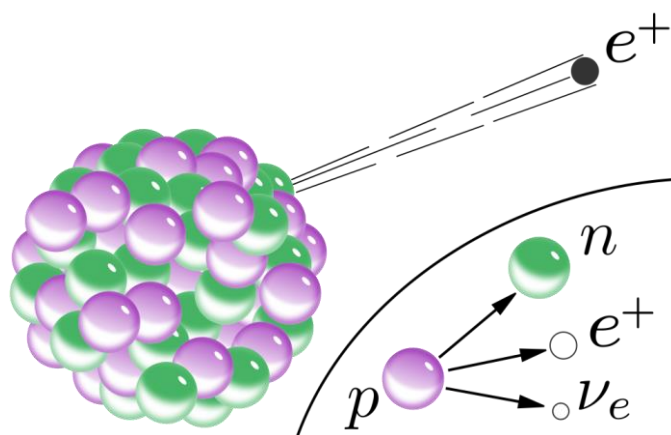


Figure 4. Schematic representation of beta plus decay and generated particles (Source: commons.wikimedia.org)

Positron emission tomography is the first technique for visualization of molecular changes in human body with the possibility for absolute quantification (Williams 2008). Recently, PET was used in combination with other imaging techniques such as CT and MRI (Weissleder & Mahmood 2001; Carney et al. 2006; Wagenknecht et al. 2013).

PET is based on the radioactive decay of nuclides which generate positrons. This decay is called beta plus. Beta plus decay occurs when, in a nucleus with too many protons, a proton decays into a neutron, a neutrino and a positron (Figure 4).

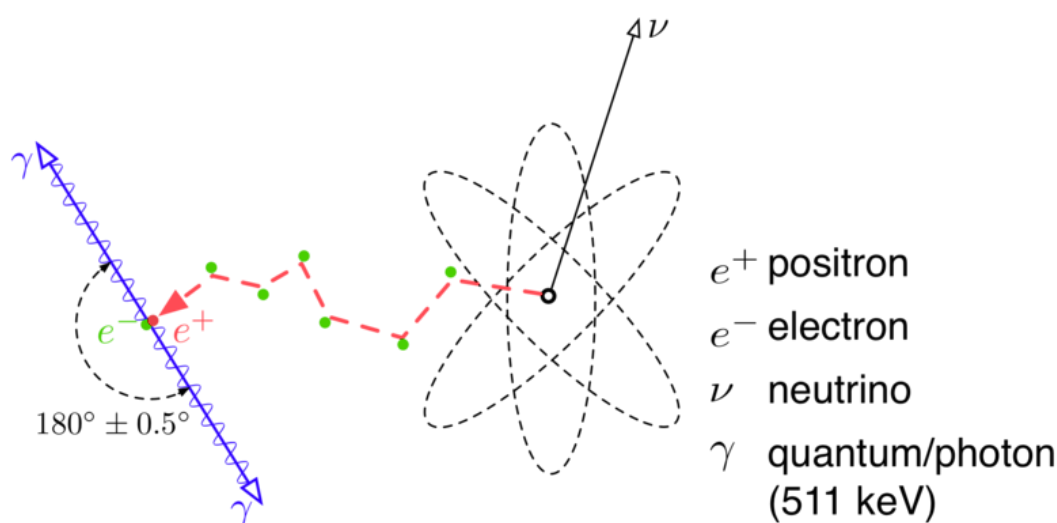


Figure 5. Positron-electron annihilation resulting complete conversion of their mass to pure energy in the form of two oppositely directed 511 keV photons (Source: commons.wikimedia.org)

In PET, a short-lived positron-emitting radionuclide is introduced into the body on a biologically active molecule. Positrons are emitted inside human tissue and move in the tissue for a short distance until they give up their kinetic energy and can interact with an electron. When a positron meets an electron, they start to interact by annihilation which produces gamma rays travelling antiparallely (Figure 5). By mapping these two 511 keV photons which arrive at the same time to PET scanner, the system can produce an image with high spatial resolution (Figure 6). Moreover, determination the position of the annihilation event is possible through the detection angle of those photons (McCarthy et al. 2009).

Hence, the biodistribution of respective radiolabeled molecules within the human body can be tracked and measured. In addition, it is possible to study interactions with specific drug targets, monitor the effect of treatment, assess pharmacokinetics (the effect of body towards the substance) and pharmacodynamics (the effect of a substance towards the body) response (Gupta et al. 2002; Workman 1995) and visualize disease pathology with PET (Dollé et al.

2008). The radiolabeled compounds are known as radiotracers. Radiotracers are designed for targeting cell membrane associated receptors, enzyme activities; and transport system. A good PET radiotracer has to possess a high binding affinity (low nanomolar or picomolar) at the target and specificity to be bound competitively on an active site of a target. Additionally, it should exhibit a high enough metabolic stability (Wadsak & Mitterhauser 2010; Knight & Wuest 2012).

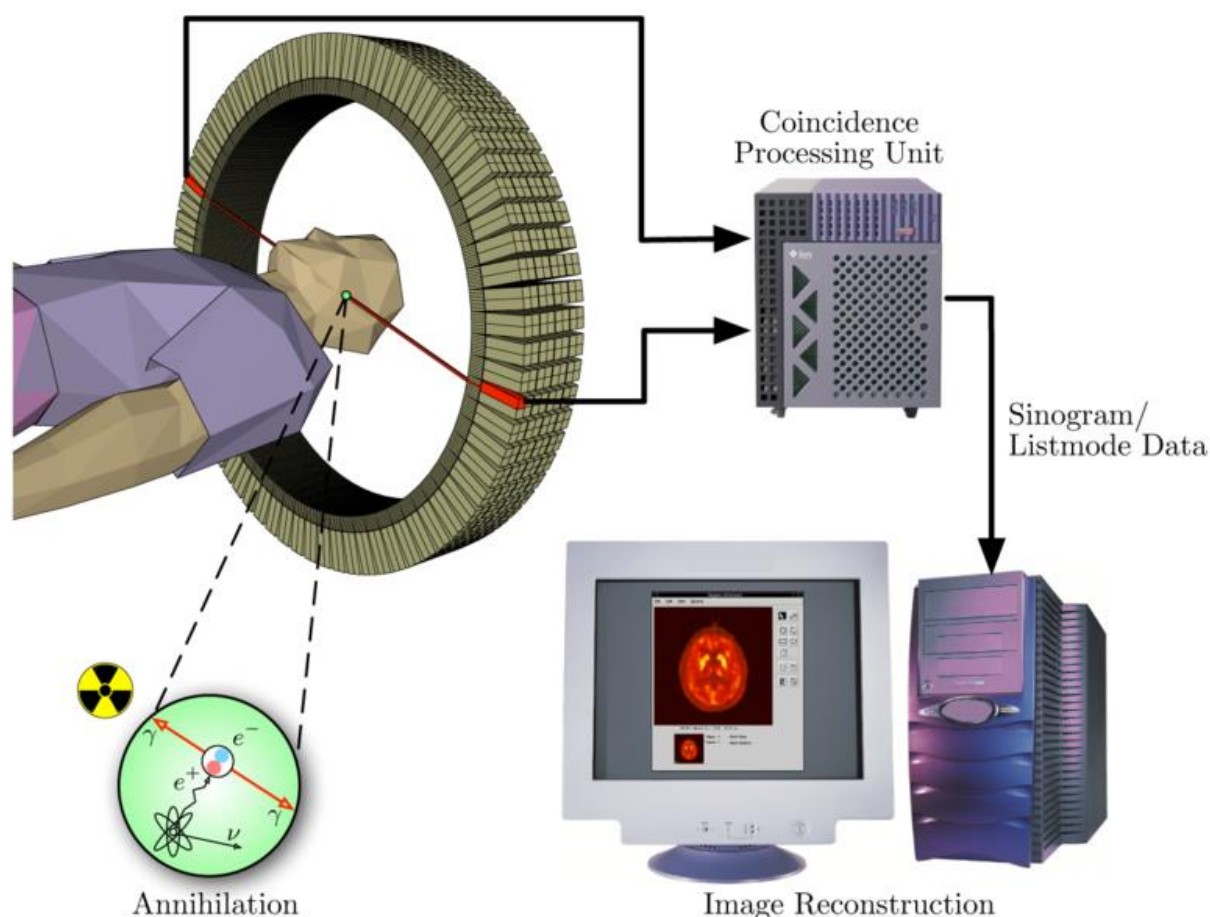


Figure 6. Schematic representation of PET imaging technique. Adapted from (Langner 2003)

One of the peculiarities of PET imaging is that few tracer molecules are enough (approximately on a nanogram scale) to produce the PET signal in sufficient intensity in the body. Only the radiation exposure must be taken into account as a potential hazard source (McCarthy et al. 2009).

1.9. PET isotopes

The choice of a suitable PET isotope for a medical-diagnostic is influenced by different independent limitations. These requirements include its half-life and chemical reactivity, the resolution of imaging, the patient exposure to the radiation, manufacturability of the PET isotope, and the desired structural design of a PET tracer which has certain physicochemical and pharmaceutical demands (Bauser & Lehmann 2012).

Table 3 shows the most typical PET radioisotopes. Fluorine-18, Carbon-11, nitrogen-13 and oxygen-15 are produced by the cyclotron. According to the short half-lives of these isotopes, an onsite cyclotron is needed. For safety reasons, the PET radiotracer must be administered into the body in the amounts that stimulate no pharmacological effect (Wadsak & Mitterhauser 2010).

Table 3. Typical PET isotopes and their properties (Dollé et al. 2008; Bauser & Lehmann 2012)

Isotope	Half-life (min)	Maximal particle energy (MeV)	Maximum molar ac- tivity (Ci/ μ mol)	Produced in
F-18	110	0.64	1712	Cyclotron
C-11	20	0.96	9215	Cyclotron
N-13	10	1.2	18430	Cyclotron
O-15	2	1.7	90960	Cyclotron
I-124	6019	2.13	31	Cyclotron
Ga-68	68	1.9	2756	Generator
Cu-62	10	2.9	138	Generator

1.10. Receptor and binding studies

Quantification and qualitatively characterization of receptors and their interactions with exogenous ligands can be carried out via radioligand binding studies. There are two major types of radioligand binding experiments, kinetic and equilibrium. Kinetic studies are used in char-

acterization of novel radioligands while the main application of equilibrium binding studies is based on measurement of the interaction between a small molecule and a protein (Pollard 2010). Different intermolecular forces such as ionic, hydrogen and van der Waals associate a ligand and its receptor and subsequently under physiological conditions the resulting ligand-receptor complex is dissociated in a reversible process (Davenport & Russell 1996). These interactions are continued repeatedly until equilibrium is reached. Equilibrium binding studies are made up to two types of experiments, saturation and competition studies (Hein et al. 2005). Saturation binding assays are used to determine dissociation constant (K_d). K_d quantifies the strength of interaction of a ligand and a receptor (Davenport & Russell 1996). Competition binding is used as a tool to evaluate selectivity of any given designated ligand for receptor sub-types. As a result of this, it is possible for us to measure density and proportion of each subtype in the tissue. In order to estimate the binding affinity of an inhibitor for a receptor we need to determine the inhibition constant which is also known as K_i . To establish a competition binding assay, a radioligand with the specified K_d value is used to determine the IC_{50} (half maximal inhibitory concentration) which will be used to estimate the K_i . In a nutshell, different concentrations of unspecified ligands are incubated with the membranes expressing a receptor and a radiolabeled agonist at a constant concentration. Plotting the specific binding as a percentage of total binding (binding in absence of competitor) versus the log concentration of competing ligand gives us competition curves (Davenport & Russell 1996; Balber 2014).

In a competition binding study, different concentrations of a nonradioactive compound compete with a constant concentration of a radiolabeled compound to bind to a receptor. IC_{50} is a concentration of the nonradioactive compound which inhibits half of binding to the radioligand. From the following equation, known as Cheng and Prusoff's equation (Yung-Chi & Prusoff 1973), dissociation constant of the a compound, K_i , is calculable from the IC_{50} :

$$K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_L}}$$

where $[L]$ is concentration of the radioligand and K_L is the association equilibrium constant for binding of the radioligand.

Chapter II:

Materials and Methods

2.1. Chemistry: From idea to target molecules

2.1.1. Ligands molecular design

The initial goal of this thesis is identifying a clinically remarkable PET radio-tracer for visualization of the beta-adrenergic receptors in CNS and PNS. Our primary investigations resulted in finding a limited list of compounds with the values of receptor binding affinity in nanomolar range and selective for β 2-ARs. A group of compounds were already labeled and evaluated, while labeling of several other compounds was recognized bothersome and expensive. For PET, the compounds containing easy labeling functional groups or short-lived positron-emitting elements were preferable. We reached a lead structure with the desirable potency (Table 4: VIEBAR11) synthesized by Tasler et al. (2010). Results from previous researches in beta adrenergic receptors and ligands, led us to find the potent analogues: Structure-activity relationship (SAR) of beta adrenergic receptor antagonists describes the basic structure and the permitted functional groups. An aromatic ring must be connected to an ethanolamine group directly or via an $-\text{OCH}_2-$ group and the amine group can be attached to a secondary substitution (Gorre & Vandekerckhove 2010; Gringauz 1997; Jucker 1971). On the other hand, in the compounds with a chiral center, (*S*)-enantiomers and (–)-enantiomers have generally higher affinity in binding with the beta-2 adrenoceptors (Mehvar & Brocks 2001; K. A. Stephenson et al. 2008; K. Stephenson et al. 2008).

Considering that the amino group in VIEBAR11 is a tertiary amine, we expected not to have high receptor binding affinity, whereas based on the Tasler's reported K_i value for the compound, we became curious to understand the paradoxon. Therefore, we intended to repeat the synthesis and binding affinity for VIEBAR11. In addition, we were interested to investigate the potency of its (*S*)-enantiomer. A group of analogues of VIEBAR11 were considered (Table 4) for the first phase of synthesis. Eight compounds (Table 4) were chosen for synthesis and finally six compounds, VIEBAR11, VIEBAR31, VIEBAR42, VIEBAR43, VIEBAR52 and VIEBAR53 were synthesized.

Table 4: Structure of the considered compounds as the potential beta-2 adrenoceptor antagonists for synthesis

Compound	Structure	IUPAC name
VIEBAR11		4-(1-(3-((9H-carbazol-4-yl)oxy)-2-hydroxypropyl)piperidine-4-carbonyl)phenyl hypofluorite
VIEBAR12		(1-(3-((9H-carbazol-4-yl)oxy)-2-hydroxypropyl)piperidin-4-yl)(4-methoxyphenyl)methanone
VIEBAR31		(S)-4-(1-(3-((9H-carbazol-4-yl)oxy)-2-hydroxypropyl)piperidine-4-carbonyl)phenyl hypofluorite
VIEBAR32		(S)-(1-(3-((9H-carbazol-4-yl)oxy)-2-hydroxypropyl)piperidin-4-yl)(4-methoxyphenyl)methanone
VIEBAR42		(S)-N-(1-(3-((9H-carbazol-4-yl)oxy)-2-hydroxypropyl)piperidin-4-yl)-4-fluorobenzamide
VIEBAR43		(S)-N-(1-(3-((9H-carbazol-4-yl)oxy)-2-hydroxypropyl)piperidin-4-yl)-4-methoxybenzamide
VIEBAR52		N-(1-(3-((9H-carbazol-4-yl)oxy)-2-hydroxypropyl)piperidin-4-yl)-4-fluorobenzamide
VIEBAR53		N-(1-(3-((9H-carbazol-4-yl)oxy)-2-hydroxypropyl)piperidin-4-yl)-4-methoxybenzamide

2.1.2. Synthesis of VIEBAR compounds

Figure 7 shows the synthesis procedure for two aimed compounds, VIEBAR11 and VIEBAR31. According to section 2.1.1, compound **1** (IUPAC name: *9H-carbazol-4-ol*) was chosen as the starting material since it would be compatible with further syntheses strategies. Compound **2** (IUPAC name: *4-(oxiran-2-ylmethoxy)-9H-carbazole*) was synthesized based on a reported procedure (Mirfeizi et al. 2014). In an empirically identical procedure, the compound **3** (IUPAC name: *(S)-4-(oxiran-2-ylmethoxy)-9H-carbazole*) was obtained in the reaction between **1** and S-oxiran-2-ylmethyl 4-methylbenzenesulfonate in the presence of potassium carbonate.

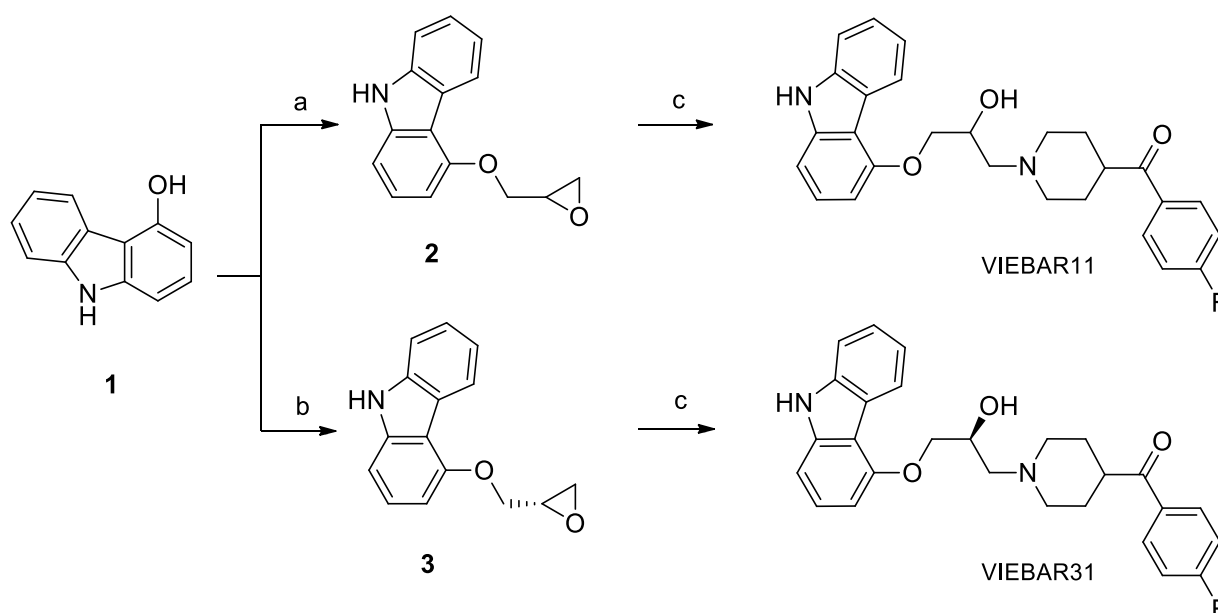


Figure 7: Reaction conditions: a) i:isopropanol/ Cs_2CO_3 ; ii: (\pm)-epichlorohydrin; 100°C, overnight b) i:isopropanol/ K_2CO_3 ; ii: S-oxiran-2-ylmethyl 4-methylbenzenesulfonate; 100°C, 16h c) isopropanol/4,4 fluoro-benzoyl piperidine, 130°C, 16h.

In Figure 8, the synthesis scheme of four novel compounds, VIEBAR42, VIEBAR43, VIEBAR52 and VIEBAR53 were described. Similar to VIEBAR11 and VIEBAR31, the synthesis started from compound **2**. 4-(N-Boc-amino) piperidine was reacted with **2** and **3** to give **4** and **5**. Using trifluoroacetic acid, the boc protection group was cleaved. Amides can be formed in reaction of acyl chlorides and amines using a suitable base and aprotic solvent at room temperature. To give novel compounds, VIEBAR42, VIEBAR43, VIEBAR52 and VIEBAR53, we used **4** and **5** as the amine moieties and 4-fluorobenzoyl chloride and 4-methoxybenzoyl chloride as the acyl chlorides. The reactions were performed in presence of triethylamine (TEA) in dichloromethane (DCM) at room temperature.

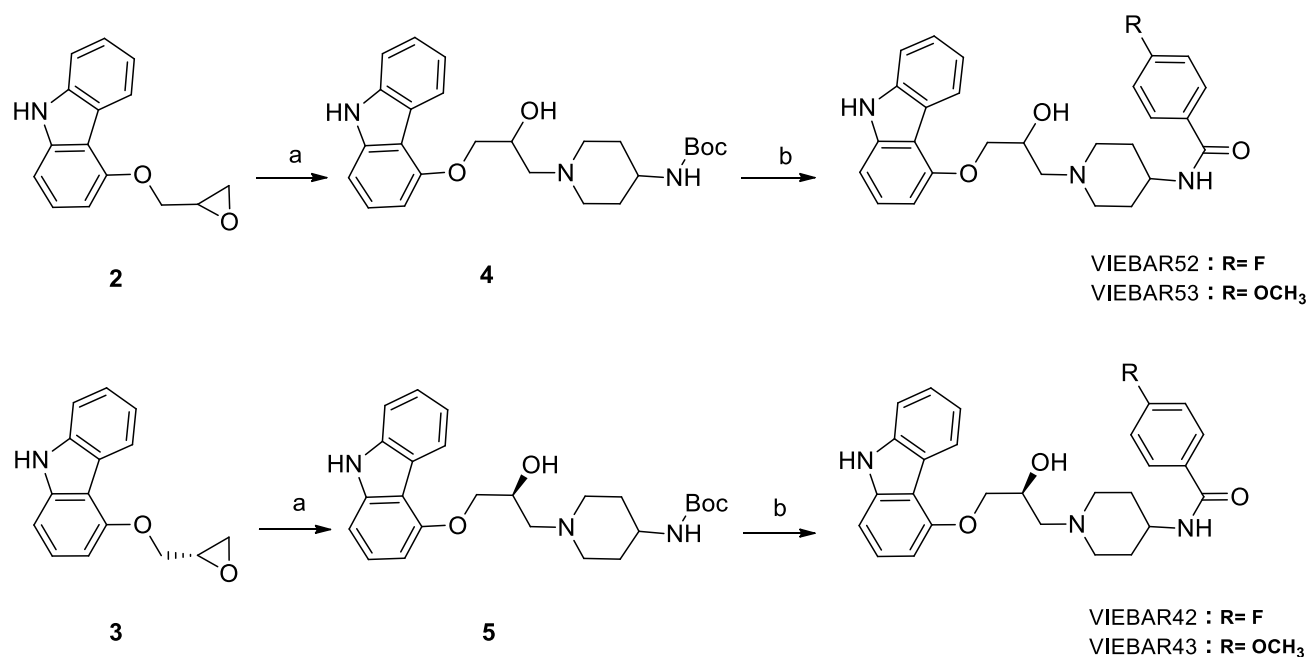


Figure 8: Reaction conditions: a) isopropanol/4-(N-Boc-amino)piperidine, 110°C, 16h; b) i: dichloromethane/TFA, rt, 16h; ii: for VIEBAR42 and VIEBAR52: triethylamine/4-methoxybenzoyl chloride, 48 hr, 38°C. ii: for VIEBAR43 and VIEBAR53: triethylamine/4-fluorobenzoyl chloride, 48 hr, 38°C.

2.1.3. Materials and instruments used for chemical synthesis

9H-Carbazol-4-ol (**1**) 95%, 2-(Chloromethyl)oxirane 99%, 2-methyl-2-propanyl 4-piperidinylcarbamate 96%, 4-fluorobenzoyl chloride 98%, 4-methoxybenzoyl chloride 99% were purchased from Sigma Aldrich. (4-Fluorophenyl)(4-piperidinyl)methanone hydrochloride (1:1) and TEA 99% were purchased from Alfa Aesar. Ethylacetate, isopropanol, dichloromethane, acetone and methanol were available with >98% purity. A Rotavapor® R-300 was used for solvent evaporation during chemical syntheses. Purity evaluation was carried out via a high-performance liquid chromatography on an Agilent Technologies 1200 Series system (Tokyo, Japan) equipped with an Agilent quaternary pump and a Raytest UV detector. NMR spectra were obtained from a 400 MHz Bruker AVANCE™ III HD-NanoBay NMR spectroscopy device. For mass spectrometry, a Bruker maXis™ high resolution mass spectrometer was used.

2.1.4. Experimental section

Compound 2

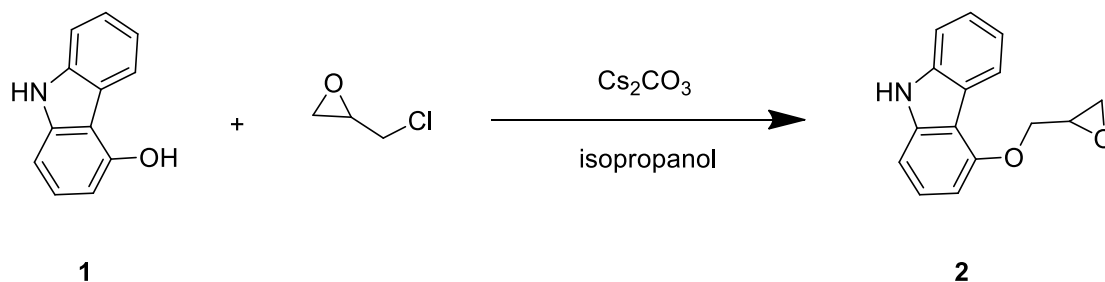


Figure 9.1. Synthesis procedure of **2**

Table 5.1. Amounts of required materials for synthesis of **2**

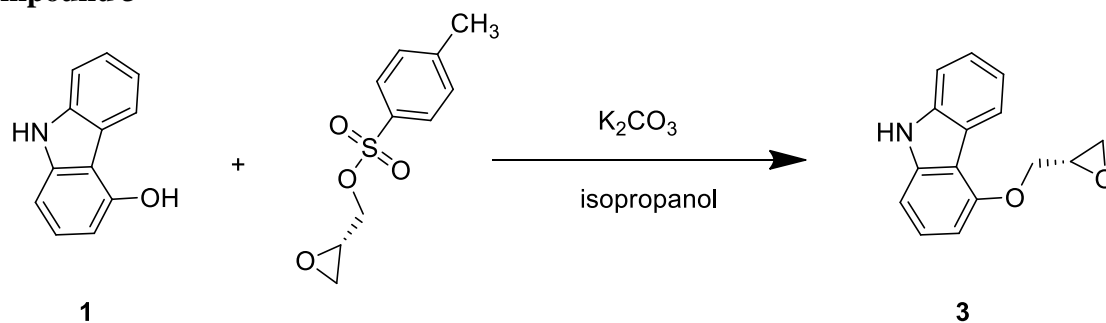
Compound	Molar mass (g/mol)	mmol /eq	Amount
Compound 1	183.21	2.74 / 1	501 mg
Epichlorohydrin	92.52	4.93 / 1.8	385 μ L (excess)
Cs ₂ CO ₃	138.21	5.48 / 2	758 mg
Isopropanol	60.1	Solvent	5 mL

To a solution of 4-hydroxycarbazole (**1**) (502 mg, 2.74 mmol, 1.0 equiv) in 5 mL of isopropanol were added 5.48 mmol (758 mg, 2 equiv) of Cs₂CO₃ and 4.93 mmol (385 μ L, 1.8 equiv) of epichlorohydrin, and the resulting mixture was stirred at 100 °C overnight. After completion of the reaction (as monitored by TLC), the solvent was evaporated in Rotavapor, and the residue was purified by silica gel chromatography (EtOAc/hexane, 2:7) to afford the compound **2** as a white solid (392 mg, 60%).

¹H NMR (400 MHz, D₂O) δ = 7.45 (td, J₁ = 7.4 Hz, J₂ = 6.0 Hz, 1 H), 7.19 (d, J = 7.7 Hz, 1 H), 7.14 (d, J₁ = 9.6 Hz, 1 H), 7.13 (dd, J₁ = 9.6 Hz, J₂ = 7.4 Hz, 1 H), 4.48 (s, 2 H).

¹³C NMR (100 MHz, D₂O) δ = 164.0, 161.6, 157.0, 138.8 (d, J = 7.5 Hz), 130.7 (d, J = 8.4 Hz), 122.5 (d, J = 2.6 Hz), 114.6 (d, J = 21.2 Hz), 113.5 (d, J = 22.3 Hz), 44.0. ¹⁹F NMR (D₂O, 282 MHz): δ = 113.4 (td, J₁ = 9.6 Hz, J₂ = 6.0 Hz, 1 F).

HRMS (HREI) Calcd for C₈H₁₀N₃F(M – HCl) + : 167.0859. Found: 167.0856.

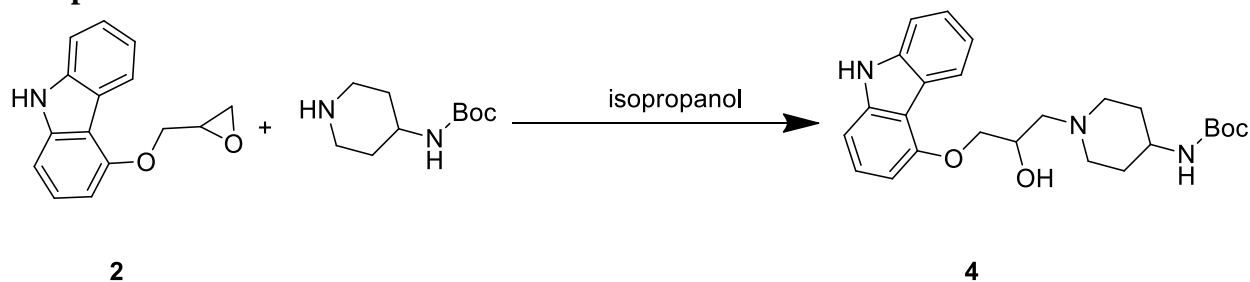
Compound 3Figure 9.2. Synthesis procedure of **3**Table 5.2. Amounts of required materials for synthesis of **3**

Compound	Molar mass (g/mol)	mmol /eq	Amount added
9H-Carbazol-4-ol	183.21	4.1 / 1	750 mg
S-Oxiran-2-ylmethyl 4-methylbenzenesulfonate	228.26	4.4 / 1.1	1000 mg
Potassium carbonate	138.21	7.23 / 1.8	1000 mg
Isopropanol	60.1	Solvent	15 mL

To a reaction tube were added sequentially 9H-carbazol-4-ol (0.75 g, 4.1 mmol), S-oxiran-2-ylmethyl 4-methylbenzenesulfonate (1.00 g, 4.4 mmol), potassium carbonate (1.00 g) and 2-butanone (15 mL). The reaction tube was sealed and heated at 100°C with stirring for 16 h. The reaction was cooled to ambient temperature, the mixture was filtered to remove salts, and the filtrate was evaporated to dryness. The residue was purified by flash chromatography on silica gel (75 g) using hexane-ethyl acetate [3:1] as the eluent. The product (0.70 g, 3.0 mmol, 73%) was isolated first, followed by a by-product which was the doubly aryloxyated compound.

^1H NMR (400 MHz, d_6 -DMSO): δ = 11.32 (s, 1 H), 8.20 (d, J = 8.0 Hz, 1 H), 7.48 (d, J = 8.0 Hz, 1 H), 7.37 (t, J = 8.0 Hz, 1 H), 7.30 (t, J = 8.0 Hz, 1 H), 7.18 (t, J = 8.0 Hz, 1 H), 7.12 (d, J = 8.0 Hz, 1 H), 6.69 (d, J = 8.0 Hz, 1 H), 4.54 (m, 1 H), 4.08 (m, 1 H), 3.55 (m, 1 H), 2.95 (m, 1 H), 2.85 (m, 1 H).

^{13}C NMR (100.59 MHz, d_6 -DMSO): δ = 154.5, 141.2, 139.0, 126.5, 124.7, 122.3, 121.6, 118.7, 111.5, 110.5, 104.3, 101.7, 68.8, 50.0, 43.8

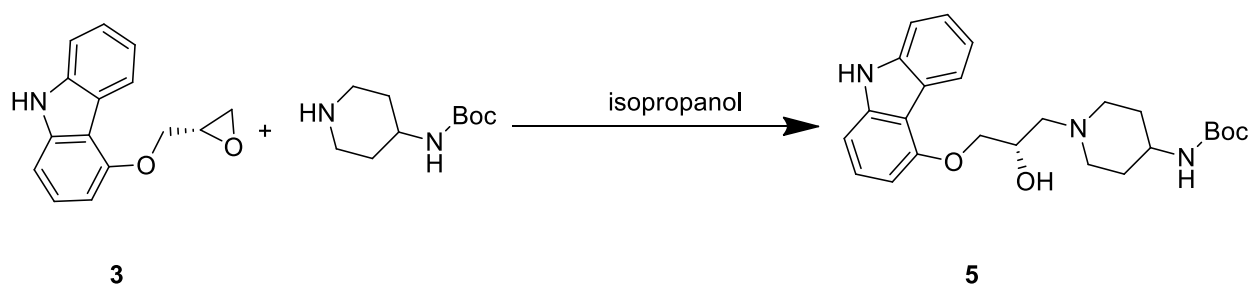
Compound 4Figure 9.3. Synthesis procedure of **4**Table 5.3. Amounts of required materials for synthesis of **4**

Compound	Molar mass (g/mol)	mmol /eq	Amount added
Compound 2	239.1	0.386 / 1	92.5 mg
4-(N-Boc-amino) piperidine	200.3	0.434 / 1.1	87 mg
Isopropanol	60.1	Solvent	1 mL

To prepare intermediate compound **4**, 87 mg (0.434 mmol, 1.1 eq) of 4-(N-Boc-amino) piperidine were added to a solution of compound **2** (92.5 mg, 0.386 mmol, 1 eq) in 1 mL of isopropanol. The mixture was stirred at 110 °C overnight and monitored by TLC. Via silica gel column chromatography, the mixture was purified (DCM:MeOH, 94:6) and after drying the solvent, 169 mg (0.384 mmol, 99%) bright olive oily was yielded.

¹H NMR (400 MHz, DMSO): δ = 1.38 (s, 9 H), 1.42 (m, 2 H), 1.67 (m, 2 H), 2.07 (m, 2 H), 2.47 (m, 1 H), 2.59 (m, 1 H), 2.90 (m, 2 H), 3.22 (m, 1 H), 4.12 (m, 1 H), 4.14 (m, 2 H), 6.68 (d, 7.9 Hz), 6.77 (d, 7.8 Hz), 7.08 (d, 7.9 Hz), 7.15 (m, 1 H), 7.27 (t, 7.9 Hz), 7.32 (m, 1 H), 7.45 (m, 1 H), 8.24 (d, 7.8 Hz), 11.25 (s, 1 H).

¹³C NMR (100 MHz, DMSO): δ = 28.2, 31.9, 47.5, 53.1, 61.3, 66.9, 70.8, 77.4, 100.4, 103.8, 110.3, 111.6, 118.5, 121.7, 122.5, 124.5, 126.5, 138.9, 141.1, 154.8, 155.0
 HRMS (HREI) Calcd for C₂₅ H₃₃ N₃ O₄ (M + H)⁺ : 440.254383 Found: 440.26.

Compound 5Figure 9.4. Synthesis procedure of **5**Table 5.4. Amounts of required materials for synthesis of **5**

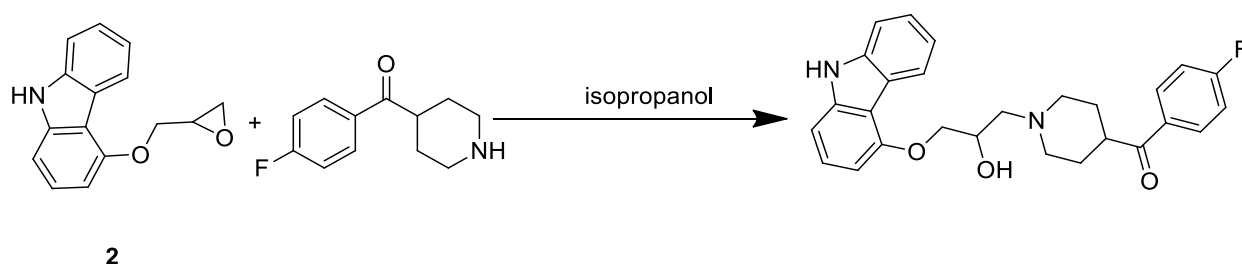
Compound	Molar mass (g/mol)	mmol /eq	Amount added
Compound 3	239.1	0.359 / 1	86 mg
4-(N-Boc-amino) piperidine	200.3	0.431 / 1.2	86 mg
Isopropanol	60.1	Solvent	1 mL

The synthesis was carried out as described for compound **4**. To a solution of compound **3** (86 mg, 0.359 mmol, 1 eq) in isopropanol, 0.431 mmol (86 mg, 1.2 eq) 4-(N-Boc-amino)piperidine were added. The mixture was heated up to 110 °C and stirred overnight. The crude product was purified by means of chromatography (10% MeOH/CHCl₃) to give compound **7** (0.325 mmol, 143 mg, 90%).

¹H NMR (400 MHz, DMSO): δ = 1.36 (s, 9 H), 1.38 (m, 2 H), 1.65 (m, 2 H), 2.05 (m, 2 H), 2.46 (m, 1 H), 2.59 (m, 1 H), 2.88 (m, 2 H), 3.19 (m, 1 H), 4.1 (m, 1 H), 4.12 (m, 2 H), 6.67 (d, 7.9 Hz), 6.74 (d, 7.8 Hz), 7.05 (d, 7.9 Hz), 7.12 (m, 1 H), 7.27 (t, 7.9 Hz), 7.32 (m, 1 H), 7.43 (m, 1 H), 8.21 (d, 7.8 Hz), 11.22 (s, 1 H).

¹³C NMR (100 MHz, DMSO): δ = 28.2, 31.9, 47.5, 53.1, 61.3, 66.9, 70.8, 77.4, 100.4, 103.8, 110.3, 111.5, 118.5, 121.7, 122.5, 124.5, 126.5, 138.9, 141.1, 154.8, 155.

HRMS (HREI) Calcd for C₂₅H₃₃N₃O₄ (M + H)⁺: 440.254383 Found: 440.2543.

VIEBAR11Figure 9.5. Synthesis procedure of **VIEBAR11**Table 5.5. Amounts of required materials for synthesis of **VIEBAR11**

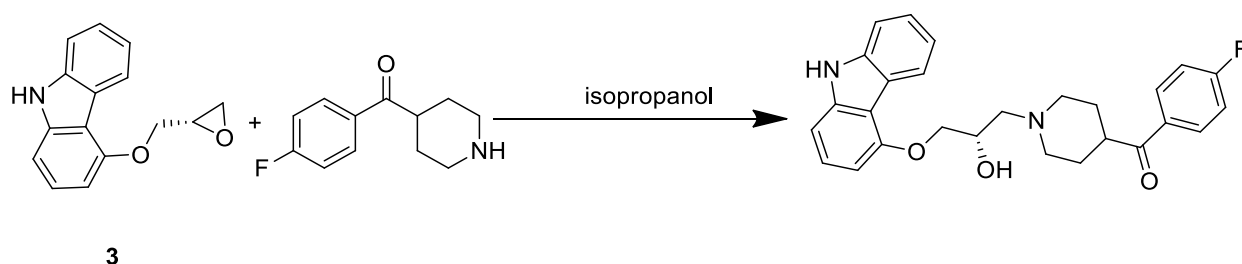
Compound	Molar mass (g/mol)	mmol /eq	Amount
Compound 2	239.2	0.313 / 1	75 mg
(4-Fluorophenyl)(piperidin-4-yl)methanone	207.2	0.376 / 1.2	78 mg
Isopropanol	60.1	Solvent	1 mL

(4-Fluorophenyl)(4-piperidiny)l)methanone was afforded as described in a reported procedure (Fakhraian & Heydary 2014). To a solution of (**2**) (75mg, 0.313 mmol, 1eq) in 1 mL of isopropanol, were added 0.376 mmol (78 mg, 1.2 eq) of 4,4 fluorobenzoyl piperidine. The reaction was carried out at 130°C overnight. After purification of the mixture via a silica gel column chromatography (DCM/MeOH 94:6), 0.264 mmol (118 mg, 84%) product was obtained. ^1H NMR (400 MHz, CDCl_3): δ = 1.88-1.98 (m, 4 H), 2.72-2.76 (m, 2 H), 2.99-2.48 (m, 2 H), 3.10 (s, 1 H), 3.19-2.24 (m, 2 H), 3.25 (m, 1 H), 4.22 (m, 1 H), 4.31 (m, 1 H), 4.32 (m, 1 H), 6.68 (d, 1 H), 7.04 (d, 1 H), 7.16 (m, 2 H), 7.24 (t, 1 H), 7.32 (t, 1 H), 7.39 (m, 2 H), 7.98 (m, 2 H), 8.18 (s, 1 H), 8.29 (d, 1 H).

^{13}C NMR (100 MHz, CDCl_3): δ = 28.7, 28.9, 43.3, 52.3, 54.7, 61.2, 65.8, 70.3, 101.1, 103.8, 110.0, 112.7, 115.8 (d, J = 21.8 Hz), 119.6, 122.5, 122.9, 124.9, 126.6, 130.8 (d, J = 9.3 Hz), 132.3 (d, J = 3 Hz), 138.7, 140.9, 155.1, 165.6 (d, J = 254.8 Hz), 200.9.

^{19}F NMR (CDCl_3 , 376 MHz): δ = -105.2 (m, 1 F).

HRMS (HREI) Calcd for $\text{C}_{27}\text{H}_{27}\text{N}_2\text{O}_3\text{F}$ ($\text{M} + \text{H}$) $^+$: 447.207847. Found: 447.2080

VIEBAR31Figure 9.6. Synthesis procedure of **VIEBAR31**Table 5.6. Amounts of required materials for synthesis of **VIEBAR31**

Compound	Molar mass (g/mol)	mmol /eq	Amount
Compound 3	239.2	0.309 / 1	75 mg
(4-Fluorophenyl)(piperidin-4-yl)methanone	207.2	0.313 / 1	65 mg
Isopropanol	60.1	Solvent	1 mL

(4-Fluorophenyl)(piperidin-4-yl)methanone (0.313 mmol, 65mg, 1eq) were added to a solution of **3** (0.309 mmol, 75 mg, 1 eq) in 1 mL of isopropanol and was heated to 130 °C and stirred overnight. The crude mixture was purified in a silica gel column (DCM/MeOH 94:6) and VIEBAR31 (0.269 mmol, 120 mg, 87%) was yielded.

¹H NMR (400 MHz, CDCl₃) δ = 1.90-1.95 (m, 4 H), 2.75 (m, 2 H), 2.49/3.01 (m, 2 H), 3.10 (broad, 1 H), 2.24/3.19 (m, 2 H), 3.23 (m, 1 H), 4.22 (m, 1 H), 4.31 (m, 1 H), 4.32 (m, 1 H), 6.69 (d, 1 H), 7.04 (d, 1 H), 7.15 (m, 2 H), 7.25 (t, 1 H), 7.31 (t, 1 H), 7.39 (m, 2 H), 7.98 (m, 2 H), 8.12 (s, 1 H), 8.29 (d, 1 H).

¹³C NMR (100 MHz, CDCl₃): δ = 28.7, 28.9, 43.3, 52.3, 54.7, 61.2, 65.8, 70.3, 101.2, 103.8, 110.0, 112.7, 115.8 (d, J = 21.7 Hz), 119.6, 122.5, 122.9, 124.9, 126.7, 130.8 (d, J = 9.2 Hz), 132.3 (d, J = 3 Hz), 138.7, 140.9, 155.2, 165.6 (d, J = 253.1 Hz), 200.9.

¹⁹F NMR (CDCl₃, 376 MHz): δ = -105.2 (m, 1 F).

HRMS (HREI) Calcd for C₂₇H₂₇N₂O₃F(M + H)⁺: 447.207847. Found: 447.2070

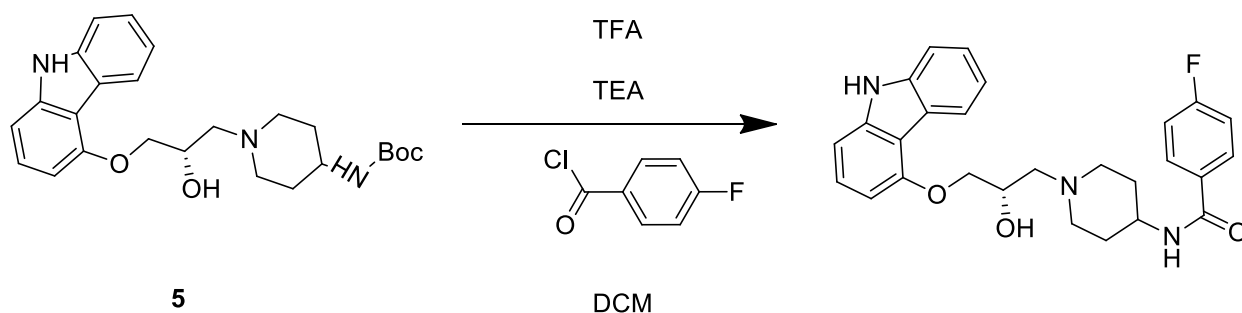
VIEBAR42

Figure 9.7. Synthesis procedure of **VIEBAR42**

Table 5.7. Amounts of required materials for synthesis of **VIEBAR42**

Compound	Molar mass (g/mol)	mmol /eq	Amount
Compound 5	439.5	0.159 / 1	70 mg
Trifluoroacetic acid	114	1.27 / 8	97 μ L
Triethylamine	101	0.477 / 3	66 μ L
4-Fluorobenzoyl chloride	158.5	0.477 / 3	60 μ L
dichloromethane	85	Solvent	2 mL

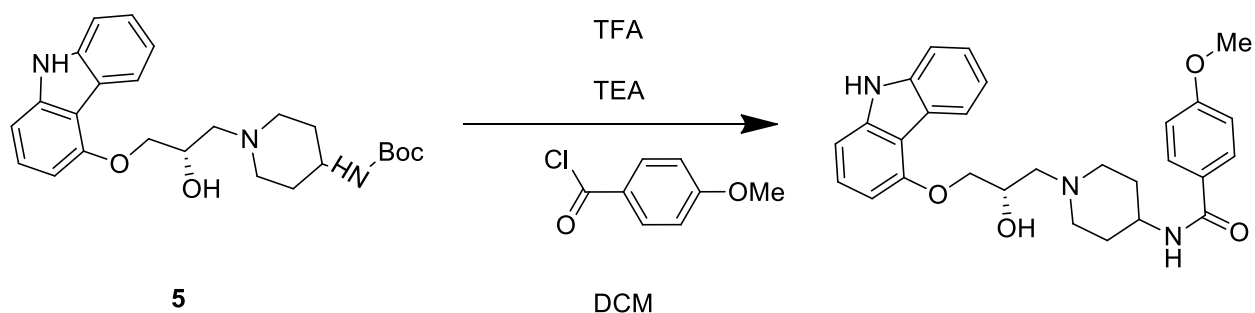
To a solution of **5** (0.159 mmol, 70 mg, 1 eq) in 2 mL of DCM were added 1.27 mmol (97 μ L, 8 eq) trifluoroacetic acid and was stirred overnight at ambient condition. 0.477 mmol (66 μ L, 3 eq) of TEA and 0.477 mmol (60 μ L, 3 eq) of 4-fluorobenzoyl chloride were added respectively and the mixture was stirred for 48 hours at 38 $^{\circ}$ C. The reaction progress was monitored by TLC. Purification of the product was undertaken twice by a silica gel column (EtOAc/MeOH, 92:8 and DCM/MeOH, 90:10). A pale white solid was collected as the product (0.12 mmol, 55 mg, 75%).

¹H NMR (400 MHz, DMSO): δ = 1.60 (m, 2H), 1.76 (m, 2H), 2.15 (m, 2H), 2.53 (m, 1H), 2.64 (m, 1H), 2.98 (m, 2H), 3.76 (m, 1H), 4.13 (m, 1H), 4.15 (m, 1H), 4.19 (m, 1H), 4.98 (m, 1H), 6.68 (m 'd', 1H), 6.96 (m, 2H), 7.06 (d, 8.0 Hz), 7.14 (m, 't', 1H), 7.26 (m, 2H), 7.28 (t, 8.0 Hz), 7.32 (m, 't', 1H), 7.43 (m, 1H), 7.90 (m, 2H), 8.24 (m, 1H), 8.25 (m, 1H), 11.23 (s, 1H).

¹³C NMR (100 MHz, DMSO-d₆): δ = 31.6, 46.9, 53.17, 53.24, 61.2, 66.9, 70.8, 100.4, 103.8, 110.3, 111.6, 115.0, 118.5, 121.7, 122.5, 124.5, 126.4, 129.9 (d, 8.9 Hz), 131.2 (d, 2.9 Hz), 138.9, 141.1, 155.0, 163.7 (d, 248 Hz), 164.5.

¹⁹F NMR (376 MHz, DMSO-d₆): δ = −109.8 (m, 1 F).

HRMS (HREI) Calcd for C₂₇H₂₈N₃O₃F (M + H)⁺ : 462.218746. Found: 462.2178.

VIEBAR43Figure 9.8. Synthesis procedure of **VIEBAR43**Table 5.8. Amounts of required materials for synthesis of **VIEBAR43**

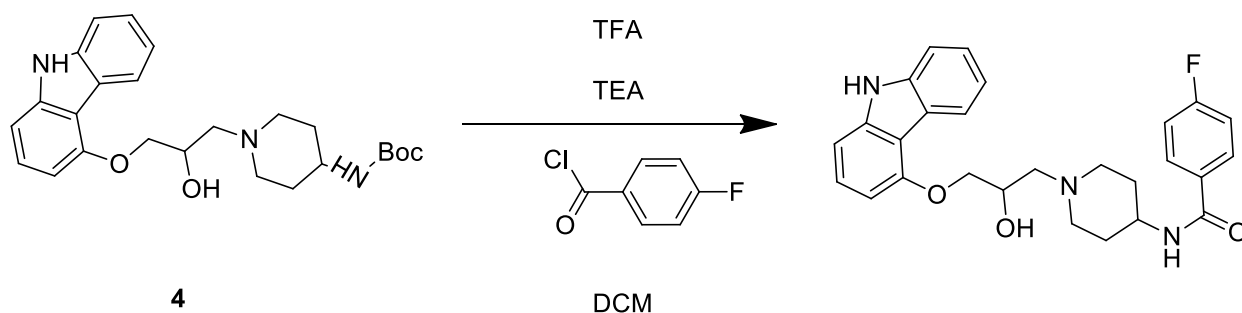
Compound	Molar mass (g/mol)	mmol /eq	Amount
Compound 5	439.5	0.136 / 1	60 mg
Trifluoroacetic acid	114	1.09 / 8	83 μ L
Triethylamine	101	0.41 / 3	57 μ L
4- Methoxybenzoyl chloride	170.6	0.41 / 3	55 μ L
Dichloromethane	85	Solvent	1 mL

To a solution of **5** (0.136 mmol, 60 mg, 1 eq) in 1 mL of DCM were added 1.09 mmol (83 μ L, 8 eq) trifluoroacetic acid and the mixture was stirred overnight at RT. 0.41 mmol (57 μ L, 3 eq) of TEA and 0.41 mmol (55 μ L, 3 eq) of 4-methoxybenzoyl chloride were added respectively and the mixture was stirred for 48 hours at 38 °C. The reaction progress was monitored by TLC. Purification of the product was undertaken twice by a silica gel column (EtOAc/MeOH, 92:8 and DCM/MeOH, 90:10) to afford 0.052 mmol (25 mg, 39%) of VIEBAR43.

^1H NMR (400 MHz, DMSO): δ = 1.61 (m, 2H), 1.76 (m, 2H), 2.56 (m, 1H), 2.68 (m, 1H), 3.00 (m, 2H), 3.19 (m, 2H), 3.35 (broad, 1H), 3.76 (m, 1H), 3.78 (s, 3H), 4.13 (m, 1H), 4.17 (m, 1H), 4.19 (m, 1H), 6.68 (d, 7.9 Hz), 6.96 (m, 2H), 7.06 (d, 8.0 Hz), 7.14 (m, 't', 1H), 7.28 (t, 8.0 Hz), 7.32 (m, 't', 1H), 7.44 (m, 'd', 1H), 7.81 (m, 2H), 8.07 (d, 7.7 Hz), 8.24 (d, 7.8), 11.24 (s, 1H).

^{13}C NMR (100 MHz, DMSO- d_6): δ = 31.5, 46.6, 53.2, 55.3, 61.1, 66.8, 70.8, 100.4, 103.8, 110.4, 111.6, 113.3, 118.5, 121.7, 122.5, 124.5, 126.5, 126.9, 129.1, 138.9, 141.1, 155.0, 161.4, 165.0.

HRMS (HREI) Calcd for $\text{C}_{28}\text{H}_{31}\text{N}_3\text{O}_4$ ($\text{M} + \text{H}^+$) : 474.2378. Found: 474.2375.

VIEBAR52Figure 9.9. Synthesis procedure of **VIEBAR52**Table 5.9. Amounts of required materials for synthesis of **VIEBAR52**

Compound	Molar mass (g/mol)	mmol /eq	Amount
Compound 4	439.5	0.22 / 1	97 mg
Trifluoroacetic acid	114	2.2 / 10	168 μL
Triethylamine	101	0.88 / 4	112 μL
4-Fluorobenzoyl chloride	158.5	0.88 / 4	122 μL
Dichloromethane	85	Solvent	2 mL

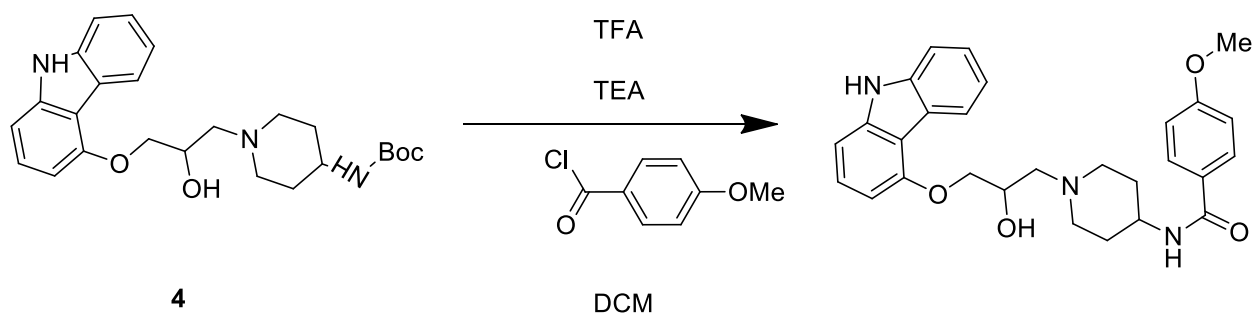
To the solution of **4** (0.22 mmol, 97 mg, 1 eq) in 2 mL of DCM were added 2.2 mmol (168 μL , 10 eq) trifluoroacetic acid and the mixture was stirred overnight at ambient condition. 0.88 mmol (112 μL , 4 eq) of TEA and 0.88 mmol (122 μL , 4 eq) of 4-fluorobenzoyl chloride were added respectively and the mixture was stirred for 48 hours at 38 °C. The reaction progress was monitored by TLC. Purification of the product was undertaken twice by a silica gel column (EtOAc/MeOH, 92:8 and dichloromethane/methanol, 90:10) to give 0.16 mmol (73 mg, 72%) of VIEBAR52.

^1H NMR (400 MHz, DMSO): δ 1.60 (m, 2H), 1.76 (m, 2H), 2.15 (m, 2H), 2.53 (m, 1H), 2.64 (m, 1H), 2.98 (m, 2H), 3.76 (m, 1H), 4.13 (m, 1H), 4.15 (m, 1H), 4.19 (m, 1H), 4.98 (m, 1H), 6.68 (m 'd', 1H), 6.96 (m, 2H), 7.06 (d, 8.0 Hz), 7.14 (m, 't', 1H), 7.26 (m, 2H), 7.28 (t, 8.0 Hz), 7.32 (m, 't', 1H), 7.43 (m, 1H), 7.90 (m, 2H), 8.24 (m, 1H), 8.25 (m, 1H), 11.23 (s, 1H).

^{13}C NMR (100 MHz, DMSO- d_6): δ = 31.6, 46.9, 53.17, 53.24, 61.2, 66.9, 70.8, 100.4, 103.8, 110.3, 111.6, 115.0, 118.5, 121.7, 122.5, 124.5, 126.4, 129.9 (d, 8.9 Hz), 131.2 (d, 2.9 Hz), 138.9, 141.1, 155.0, 163.7 (d, 248 Hz), 164.5

^{19}F NMR (376 MHz, DMSO- d_6): δ = -109.8 (m, 1 F).

HRMS (HREI) Calcd for $\text{C}_{27}\text{H}_{28}\text{N}_3\text{O}_3\text{F}$ ($\text{M} + \text{H}$) $^+$: 462.218746. Found: 462.2177.

VIEBAR53Figure 9.10. Synthesis procedure of **VIEBAR53**Table 5.10. Amounts of required materials for synthesis of **VIEBAR53**

Compound	Molar mass (g/mol)	mmol /eq	Amount
Compound 4	439.5	0.136 / 1	60 mg
Trifluoroacetic acid	114	1.09 / 8	83 μ L
Triethylamine	101	0.41 / 3	57 μ L
4-Fluorobenzoyl chloride	170.6	0.41 / 3	55 μ L
Dichloromethane	85	Solvent	2 mL

Following the same procedure as for VIEBAR43, compound VIEBAR53 was isolated (0.07 mmol, 34 mg, 52%). ^1H NMR (400 MHz, DMSO): δ = 1.61 (m, 2H), 1.76 (m, 2H), 2.56 (m, 1H), 2.68 (m, 1H), 3.00 (m, 2H), 3.19 (m, 2H), 3.35 (broad, 1H), 3.76 (m, 1H), 3.78 (s, 3H), 4.13 (m, 1H), 4.17 (m, 1H), 4.19 (m, 1H), 6.68 (d, 7.9 Hz), 6.96 (m, 2H), 7.06 (d, 8.0 Hz), 7.14 (m, 't', 1H), 7.28 (t, 8.0 Hz), 7.32 (m, 't', 1H), 7.44 (m, 'd', 1H), 7.81 (m, 2H), 8.07 (d, 7.7 Hz), 8.24 (d, 7.8), 11.24 (s, 1H).

^{13}C NMR (100 MHz, DMSO- d_6): δ = 31.5, 46.6, 53.2, 55.3, 61.1, 66.8, 70.8, 100.4, 103.8, 110.4, 111.6, 113.3, 118.5, 121.7, 122.5, 124.5, 126.5, 126.9, 129.1, 138.9, 141.1, 155.0, 161.4, 165.0.

HRMS (HREI) Calcd for $\text{C}_{28}\text{H}_{31}\text{N}_3\text{O}_4$ ($\text{M} + \text{H}^+$): 474.2378. Found: 474.2384.

2.1.5. Purity evaluation

Analytical HPLC was conducted on a non-polar C-18 Agilent column (Eclipse plus, 4.6 x 100 mm) as stationary phase and a flow rate of 2 mL/min. 100 μ M solutions of the compounds in DMSO and acetonitrile (DMSO:ACN 1:9) were prepared and injected into the system. The eluent system was begun with H₂O:ACN:TFA (95:5:0.1) for two min and then gradually changed to H₂O:ACN:TFA (5:95:0.1) over next 13 min. Chromatograms by which the retention times and purity percent were calculated, obtained for 15 min at the wavelength of 254 nm while the reference wavelength was 450 nm.

2.2. Pharmacology: Receptor binding studies

2.2.1. Materials and instruments

Novel compounds VIEBAR11, VIEBAR31, VIEBAR42, VIEBAR43, VIEBAR52 and VIEBAR53 were synthesized and purified successfully. The membranes expressing human adrenergic β 1 (CHO-K1 cell line, B_{Max} = 17 pmol/mg), β 2 (HEK293-EBNA cell line, B_{Max} = 3.8 pmol/mg) and β 3 (CHO-K1, B_{Max} = 980 fmol/mg) receptors were purchased from PerkinElmer. [³H](–)-CGP12177 (molar activity = 1284 GBq/mmol), the radioligand which was used for all binding assays was prepared commercially from PerkinElmer, too. HEPES (99.5%), Sodium chloride (99%), EGTA (97%), Tris(hydroxymethyl)aminomethane (99.8%), ICI 118,551 hydrochloride (98%), (–)-propranolol (98%) and EDTA 99% were bought from Sigma-Aldrich. Moreover, magnesium chloride hexahydrate (99%) and hydrogen chloride 10% were purchased from Merck and Ultimate Gold™ liquid scintillation cocktail from Perkin Elmer was available.

A Brandel® M-36 Cell Harvester (U.S.A) and Whatman™ GF/B and GF/C filters were used to perform rapid filtration. Radioactivity was determined via a Hidex 300 SL Automatic TDCR Liquid Scintillation Counter (Finland). Eppendorf Research® plus pipettes were used for manual liquid handling while the vials and tubes were also purchased from Eppendorf (Germany). We used Whatman™ GF/B and GF/C filters for rapid filtration process.

2.2.2. Sample preparation

Competitors: VIEBAR compounds

A 1 mM solution of each of the VIEBAR compound was prepared (Table 6). To give 100 μ M solution, the 1 mM solution was diluted 1:10 in DMSO by taking 100 μ L of the 1 mM VIEBAR solution and addition of 900 μ L of DMSO. Concentrations 10 μ M, 1 μ M and 100 nM were prepared in a same procedure.

Table 6. Composition of the competitor solutions

Compound	Molecular weight (g/mol)	Taken amount (μ g)	Solvent	Solvent ad- ded (μ L)	Final con- centration
VIEBAR11	447	198	DMSO	443	1 mM
VIEBAR31	447	750	DMSO	1220	1 mM
VIEBAR42	461	474	DMSO	1028	1 mM
VIEBAR43	474	424	DMSO	894	1 mM
VIEBAR52	461	470	DMSO	1017	1 mM
VIEBAR53	474	175	DMSO	369	1 mM

Moreover, 10 μ M solutions of ICI 118,551 and (-)-propranolol using for determination of non-specific binding were prepared.

Membrane

400 μ L of the frozen membrane expressing human β 1-ARs with a concentration of 10 μ g/ μ L were thawed and aliquoted into eleven 36 μ L aliquots, resulting in a protein concentration of 360 μ g per aliquot. Based on the manufacturer recommendation (Appendix: Technical data sheet of Perkin Elmer products), the aliquots were immediately stored at -80 $^{\circ}$ C.

Protein concentration of the membrane expressing human β 2-ARs was 1 μ g/ μ L. A 400 μ L frozen aliquot of the membranes was divided into eleven aliquots as mentioned above for human β 1-AR. Concentration of each one of 36 μ L β 2-AR aliquot was 36 μ g/aliquot.

A same process was performed for the 400 μL frozen aliquot of membrane expressing β_3 -ARs. Protein concentration of the membrane lot was 2 $\mu\text{g}/\mu\text{L}$ in which 36 μL aliquot was 72 μg protein.

We will refer later that based on the protocol of the radioligand binding assay, membrane pellets were diluted in the assay buffers.

Buffers

Perkin Elmer[®] recommended for each type of β -AR membrane binding assay an assay buffer for diluting the competitors, membranes and radioligand and a wash buffer for the harvesting procedure. All buffers have physiological pH of 7.4. Wash buffers were cooled down in the fridge.

Assay buffer for β_1 -AR assay was composed from 50 mM Tris-HCl, 150 mM sodium chloride and 0.02% ascorbic acid while the wash buffer consists of only 50 mM Tris-HCl (pH of 7.4). For 1 L assay buffer solution, we took 6.05 g of Tris(hydroxymethyl)aminomethane which was dissolved in distilled water. The pH of solution was regulated by adding hydrogen chloride 10% dropwise until reached to 7.4. In case of assay buffer, then 8.77 g of sodium chloride and 200 mg of ascorbic acid were added to the solution. Then the solution was transferred into a volumetric flask and was filled with distilled water until the volume was 1 L accurately.

For β_2 -AR assay buffer we prepared an aquatic solution of 50 mM Tris-HCl, 20 mM of magnesium chloride and 2 mM EGTA. For preparation of 50 mM Tris-HCl pH 7.4 see β_1 -AR assay buffer. Then 4.06 g of MgCl_2 and 760 mg of EGTA were added and the volume of the solution was increased to 1 L by adding distilled water in a volumetric flask. We used an identical wash buffer for both β_1 -AR and β_2 -AR assays.

Assay buffer of β_3 -AR assay consists of 25 mM HEPES, 1 mM EDTA and 0.5% bovine serum albumin (BSA). 5.95 g Hepes was dissolved in 500 mL distilled water whose pH then reached to 7.4 by adding sodium hydroxide 1 M dropwise. 292 mg EDTA and 5 g BSA were added to the solution. Since BSA is soluble in water at room temperature only slightly, the temperature was increased up to 45 $^{\circ}\text{C}$. The solution was poured into a 1 L volumetric flask

and was filled with distilled water up to 1 L. An ice-cold buffer was used for washing consist of 10 mM (for 1 L, 2.38 g) Hepes and 500 mM (for 1 L, 29.22 g) sodium chloride.

Radioligand

[5,7- ^3H] 4-[3-[(1,1-dimethylethyl)amino]2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one hydrochloride (Figure 10) known as [5,7- ^3H] (-)-CGP-12177; molar activity 1284 GBq/mmol, was the selected radioligand in this study; which is a non-selective ligand for β -ARs that binds to all β -AR subtypes given in table 7.

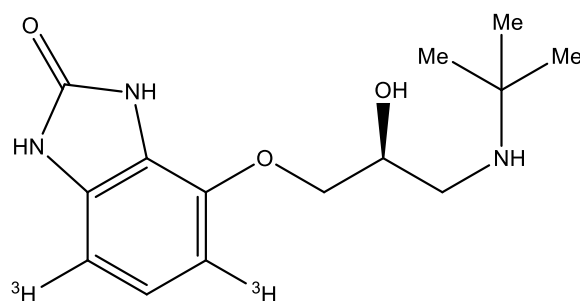


Figure 10. Structure of [5,7- ^3H] (-)-CGP-12177

For the radioligand, [^3H]-CGP 12177, the activity concentration of 37 MBq/ml, was given by the manufacturer (Appendix: Technical data sheet of Perkin Elmer products).

Table 7. The K_D values for [^3H]-CGP 12177 in the cell lines for the β -ARs

β -AR subtype	K_D (nM)	Cell-line	Reference
β_1 -AR	0.42	CHO-K1	(Baker 2010; Baker 2005)
β_2 -AR	0.17	CHO-K1	(Baker 2010; Baker 2005)
β_3 -AR	109.2	CHO-K1	(Baker 2010; Baker 2005)

Binding assays for β 1- and β 2-AR membranes were established in a previous work and the final concentration of the radioligand was adjusted to 1 nM (Weber 2016). Hence, we added 0.7 μ L of [3 H]-CGP 12177 to 2 mL of assay buffers to have the concentration of 10 nM. According to volume ratio of radioligand to whole assay which was 1:10, the final concentration of radioligand resulted 1 nM. The calculations are provided in the following:

$$C = \frac{C_a}{A_s} = \frac{0.037 \text{ GBq/mL}}{1283.9 \text{ GBq/mmol}} = 2.88 \times 10^{-5} \text{ mmol/mL} = 28818 \text{ nM}$$

$$\frac{2000 \mu\text{L} \times 10 \text{ nM}}{28818 \text{ nM}} = 0.7 \mu\text{L}$$

where C_a is the activity concentration and A_s is molar activity; both given by manufacturer (Balber 2014).

The final concentration of radioligand in β 3-AR binding assay was 5 nM (Weber 2016). For this purpose, 3.5 μ L of [3 H]-CGP 12177 was added to 2 mL of β 3-AR binding assay buffer to result 5 nM according to the volumes of the components of the assay.

2.2.3. Radioligand binding assay

Considering the recommended protocols from Perkin Elmer, we established three different competition binding assays for three β -AR subtypes. For β 1-AR and β 3-AR binding assays, the experiments were performed in 500 μ L whereas for β 2-AR binding studies we set up the assay in volume of 100 μ L. Tables 8, 9 and 10 show the compositions of the samples in different assays. In these assays, VIEBAR compounds were taken and tested in five concentrations (according to the limited membrane stock, in some assays three or four concentrations were analyzed) ranging from 100 nM to 1 mM all in triplicate.

Total binding is the summation of the specific and non-specific binding. As (Mendel & Mendel 1985) define, nonspecific binding is “*binding that was not displaceable by excess concentrations of ligands that bound to the physiological receptor*”. Total specific and nonspecific binding of radioligand were determined in absence of competitor and in presence of a competitor with high affinity for the β -AR, respectively. For total specific binding evaluations of radioligand, we used dimethyl sulfoxide (DMSO) as the solvent of all test compounds in the assays, instead of the VIEBAR competitors in a same volume. Non-specific

binding of radioligand for β_2 -AR assay was determined by using 1 mM ICI 118,551 as a β_2 -AR selective antagonist. For β_1 -AR and β_3 -AR assays, non-specific binding of [3 H]-CGP 12177 were evaluated with a non-selective β -AR agonist, (-)-propranolol, in concentration of 1 mM (Tables 8, 9 and 10). In all experiments, triplicated samples were made for total specific and non-specific binding determination.

Radioligand binding studies were performed as is demonstrated in Figure 11. After mixture of membrane suspensions expressing β -ARs, [3 H]-CGP 12177 and test compounds were prepared, they were incubated at 27 °C in a waterbath to avoid temperature variation (Table 11). Immediately after radioligand-receptor-Binding were reached to equilibrium (for incubation times, see Table 11), ice cold buffer were added and Rapid filtration through a Brandel harvester using glassfiber filters following by determination radioactivity of the filters were carried out.

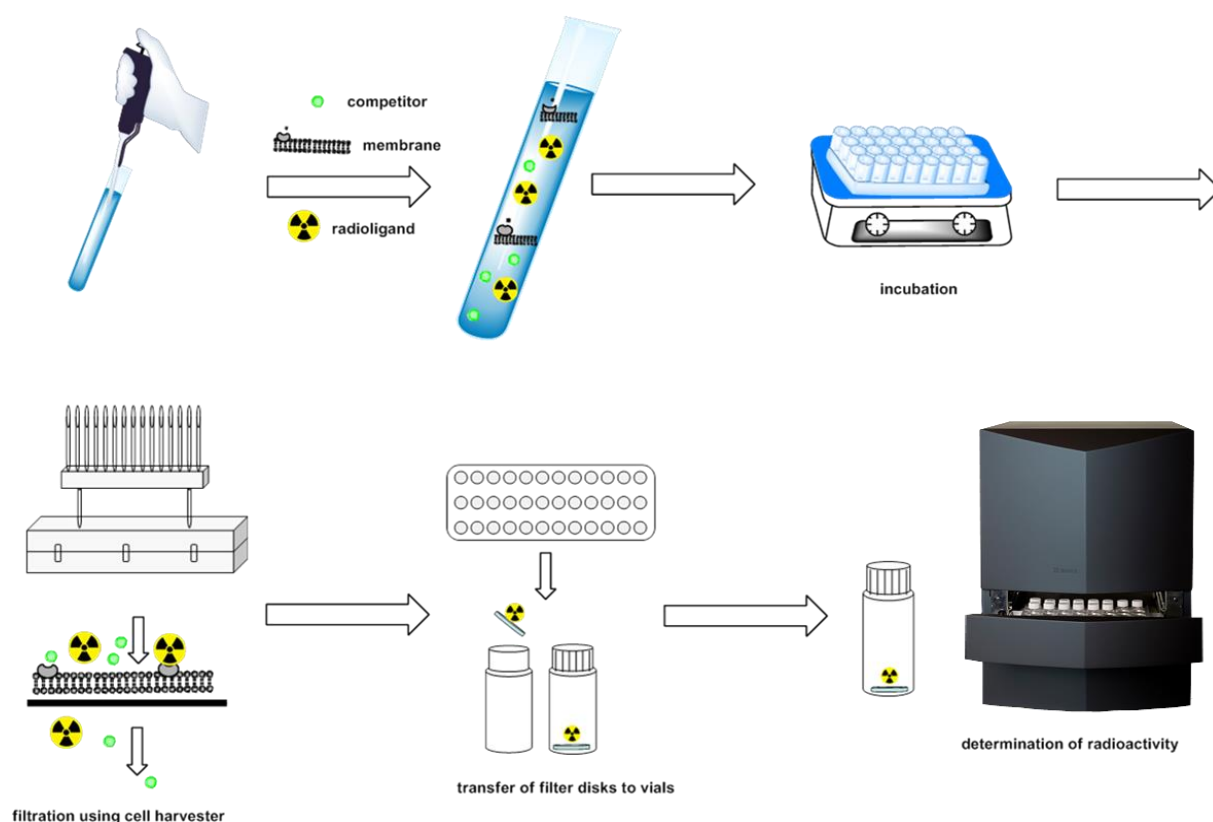


Figure 11. Scheme of radioligand binding assay; adapted from (Balber 2014)

In the case of β_1 -AR assays, we prepared 36 samples with a volume of 500 μ L (Table 8), which include three control probes as total binding samples and three nonspecific binding samples containing 5 μ L of 1 mM (-)-propranolol (concentration in assay: 10 μ L). Additionally, up to five different concentrations of two test compounds (each in triplicate) constitute

the rest of samples. Briefly, 150 μL (10 $\mu\text{g}/\mu\text{L}$ of protein, diluted 1:150 v/v in Tris-HCl buffer) of $\beta_1\text{AR}$ -containing pellets were added to 50 μL of 10 nM [^3H]-CGP 12177 ($K_{\text{d}\beta_1} = 0.42$ nM), 5 μL of different concentrations of the VIEBAR compounds (dissolved and diluted with DMSO) and 295 μL of 50 mM Tris-HCl buffer which each one performed in triplicate and incubated for 1 hour at 27 $^{\circ}\text{C}$. The samples were filtered rapidly through Whatman GF/B filter followed by twice washing via 4 mL of same buffer. The membrane-containing filters were punched into scintillation vials while 4 mL of the liquid scintillation cocktail was added to each of them. The samples were shaken for 30 min and then were placed in a liquid scintillation counter (Hidex 300 SL Automatic TDCR Liquid Scintillation Counter) to determine radioactivity (counts per minute). For nonspecific binding measurement, 5 μL of 10 μM (–)-propranolol was used. The difference between total and nonspecific binding was determined as the specific binding.

Table 8. Assay components of beta-1 adrenergic receptor: Samples 1-3 total specific binding; 4-6 non-specific binding; 7-21 and 22-36 competitors

Samples	Compound	Vol.	Conc.	[^3H]-CGP 12177 Vol. (Conc.)	β_1 -AR Membrane	Buffer
1-3	DMSO	5 μL	-	50 μL (10 nM)	150 μL	295 μL
4-6	(–)- propranolol	5 μL	10 μM	50 μL (10 nM)	150 μL	295 μL
7-9	VIEBARX	5 μL	1 nM	50 μL (10 nM)	150 μL	295 μL
10-12	VIEBARX	5 μL	10 nM	50 μL (10 nM)	150 μL	295 μL
13-15	VIEBARX	5 μL	100 nM	50 μL (10 nM)	150 μL	295 μL
16-18	VIEBARX	5 μL	1 μM	50 μL (10 nM)	150 μL	295 μL
19-21	VIEBARX	5 μL	10 μM	50 μL (10 nM)	150 μL	295 μL
22-24	VIEBARY	5 μL	1 nM	50 μL (10 nM)	150 μL	295 μL
25-27	VIEBARY	5 μL	10 nM	50 μL (10 nM)	150 μL	295 μL
28-30	VIEBARY	5 μL	100 nM	50 μL (10 nM)	150 μL	295 μL
31-33	VIEBARY	5 μL	1 μM	50 μL (10 nM)	150 μL	295 μL
34-36	VIEBARY	5 μL	10 μM	50 μL (10 nM)	150 μL	295 μL

Radioligand binding experiments for β 2-AR were accomplished in a similar procedure. 89 μ L of the human β 2-ARs (1 μ g/ μ L of protein diluted 1:45 v/v in 50 mM Tris-HCl buffer enriched with 20 mM MgCl_2 and 2 mM EGTA at pH 7.4) were incubated for ninety minutes at 27 °C in presence of 10 μ L of 10 nM [^3H]CGP12177 ($K_{\text{D}2} = 0.17$ nM) and 1 μ L of different concentrations of the VIEBAR compounds (dissolved and diluted with DMSO). 1 μ L of 1mM solution ICI 118,551 (final concentration: 10 μ M) was used for non-specific binding. The samples were triplicated.

Table 9. Assay components of beta-2 adrenergic receptor: Samples 1-3 total specific binding; 4-6 non-specific binding; 7-21 and 22-36 competitors

Samples	Compound	Vol.	Conc.	[^3H]-CGP 12177 Vol. (Conc.)	β 2-AR Membrane
1-3	DMSO	1 μ L	-	10 μ L (1 nM)	89 μ L
4-6	ICI 118,551	1 μ L	10 μ M	10 μ L (1 nM)	89 μ L
7-9	VIEBARX	1 μ L	1 nM	10 μ L (1 nM)	89 μ L
10-12	VIEBARX	1 μ L	10 nM	10 μ L (1 nM)	89 μ L
13-15	VIEBARX	1 μ L	100 nM	10 μ L (1 nM)	89 μ L
16-18	VIEBARX	1 μ L	1 μ M	10 μ L (1 nM)	89 μ L
19-21	VIEBARX	1 μ L	10 μ M	10 μ L (1 nM)	89 μ L
22-24	VIEBARY	1 μ L	1 nM	10 μ L (1 nM)	89 μ L
25-27	VIEBARY	1 μ L	10 nM	10 μ L (1 nM)	89 μ L
28-30	VIEBARY	1 μ L	100 nM	10 μ L (1 nM)	89 μ L
31-33	VIEBARY	1 μ L	1 μ M	10 μ L (1 nM)	89 μ L
34-36	VIEBARY	1 μ L	10 μ M	10 μ L (1 nM)	89 μ L

Through rapid filtration using Whatman glass microfiber GF/C filter, the samples were subsequently washed with cold Tris buffer (50 mM pH 7.4) and punched into the scintillation vials. 4 mL of Liquid scintillation cocktail was added to each sample followed by shaking. The counting was conducted by scintillation spectrometer (Hidex 300 SL Automatic TDCR Liquid Scintillation Counter).

β 3-AR binding assay was established same as β 1-AR binding assay. The differences were in the type of membrane and buffers (as mentioned in section 2.2.2), concentration of radioligand (50 nM for β 3-AR binding assay) and incubation time (30 min).

Table 10. Assay components of beta-3 adrenergic receptor: Samples 1-3 total specific binding; 4-6 non-specific binding; 7-21 and 22-36 competitors

Samples	Compound	Vol.	Conc.	[³ H]-CGP 12177 Vol. (Conc.)	β3-AR Membrane	Buffer
1-3	DMSO	5 μL	-	50 μL (50 nM)	150 μL	295 μL
4-6	(-)-propranolol	5 μL	10 μM	50 μL (50 nM)	150 μL	295 μL
7-9	VIEBARX	5 μL	1 nM	50 μL (50 nM)	150 μL	295 μL
10-12	VIEBARX	5 μL	10 nM	50 μL (50 nM)	150 μL	295 μL
13-15	VIEBARX	5 μL	100 nM	50 μL (50 nM)	150 μL	295 μL
16-18	VIEBARX	5 μL	1 μM	50 μL (50 nM)	150 μL	295 μL
19-21	VIEBARX	5 μL	10 μM	50 μL (50 nM)	150 μL	295 μL
22-24	VIEBARY	5 μL	1 nM	50 μL (50 nM)	150 μL	295 μL
25-27	VIEBARY	5 μL	10 nM	50 μL (50 nM)	150 μL	295 μL
28-30	VIEBARY	5 μL	100 nM	50 μL (50 nM)	150 μL	295 μL
31-33	VIEBARY	5 μL	1 μM	50 μL (50 nM)	150 μL	295 μL
34-36	VIEBARY	5 μL	10 μM	50 μL (50 nM)	150 μL	295 μL

A liquid scintillation counter (beta counter) was used for radioactivity determination. The efficiency of device was provided by the manufacturer up to 72%. Counting was carried out one minute per sample and the radioactivity was measured in counts per minute (cpm).

Table 11. Assay parameters of beta adrenergic receptors binding assays

Assay parameter	β1-AR	β2-AR	β3-AR
Radioligand	[³ H]-CGP 12177	[³ H]-CGP 12177	[³ H]-CGP 12177
Non-specific binding compound	(-)-propranolol	ICI 118,551	(-)-propranolol
Incubation time	60 min	90 min	30 min
Temperature	27 °C	27 °C	27 °C
Filter type	GF/B	GF/C	GF/B

2.2.4. Data analysis

The amount of radioligand ($[^3\text{H}]$ -CGP 12177) bound to respective receptors was determined by measuring radioactivity on the filter pieces. Competitive binding curves were generated using the Graphpad Prism Software to obtain IC₅₀ values.

In present study, competition curves were analyzed by computer assisted program, GraphPad Prism® 7. IC₅₀ of test compounds were determined through a non-linear regression analysis of membrane bound radioactivity expressed in cpm and the concentration of the competitor. In Figure 12, the procedure of software analysis is summarized.

In this software, we chose an X-Y function from the initial menu. Then, the concentrations were entered as the X axis values and the cpm values were imported as the Y axis. By clicking on the «Analyze» icon, a new window was opened in which «transform» function was chosen to convert X axis values as provided in the following: $X=\log X$

We used an already defined function in the software, «log(inhibitor) vs response – variable slope» to plot the competition curves and calculate the IC₅₀ values.

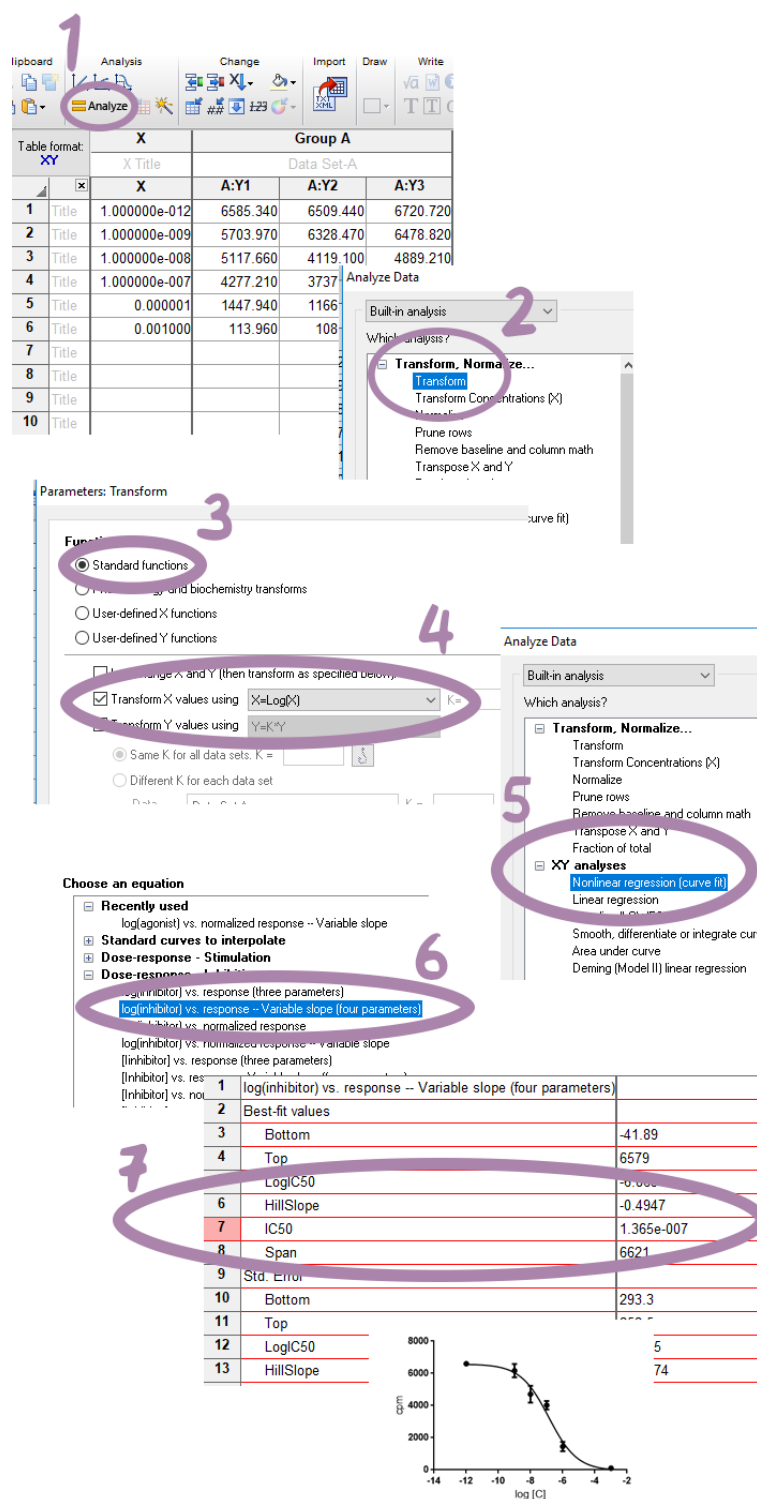


Figure 12. Data analysis procedure with Prism Graphpad 7.0 for sketching competition curves

Chapter III:

Results and Discussions

3.1. Synthesis of VIEBAR compounds

Compound **2** was synthesized from 9H-carbazol-4-ol (**1**) and 2-(chloromethyl)oxirane in 60% yield (Mirfeizi et al. 2014). Cs_2CO_3 as a base is used to deprotonate the alcohol, 9H-carbazol-4-ol. Proposed mechanism of the reaction is that two concerted $\text{S}_\text{N}2$ reactions occur during the ring opening and closing of the epoxide group (Figure 13).

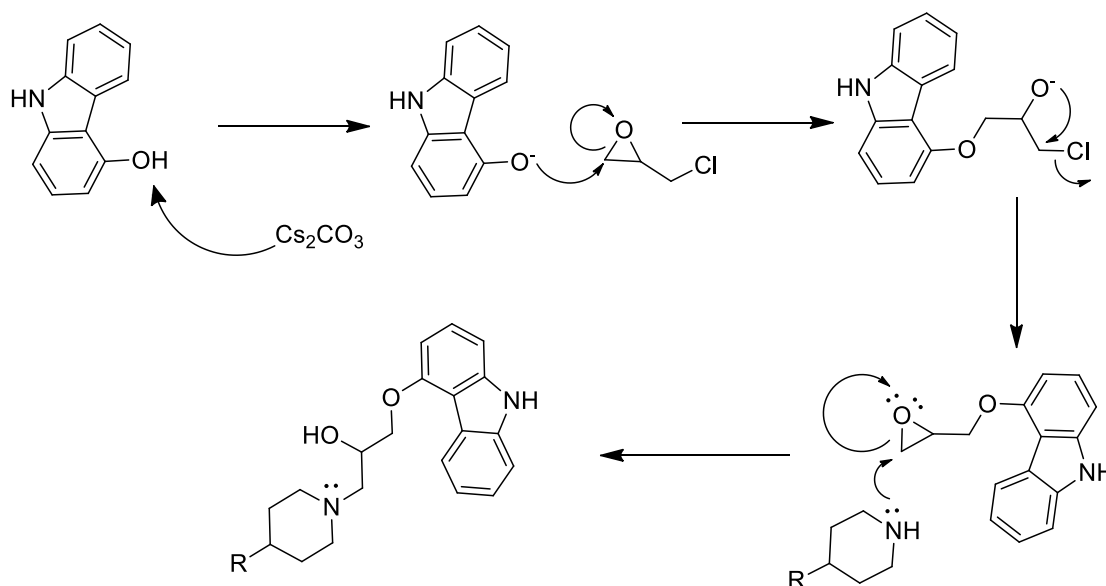


Figure 13. Proposed mechanism of synthesis of VIEBAR compounds

Synthesis of **3** was conducted in reaction between **1** and S-oxiran-2-ylmethyl 4-methylbenzenesulfonate in a similar reaction mechanism to **2**. (4-Fluorophenyl)(4-piperidinyl)methanone in a basic media opened the epoxide ring in reaction with **2** and connected from the less substituted carbon as we expect in $\text{S}_\text{N}2$ reactions. The reaction of (4-fluorophenyl)(4-piperidinyl)methanone with the epoxides in **2** and **3** proceeded to give the target compounds VIEBAR11 and VIEBAR31 in yields of 84% (50% overall yield) and 87% (52% overall yield), respectively. Reaction between **2** and 4-(N-Boc-amino)piperidine gave **4** in essentially quantitative yield. Removal of Boc group using trifluoroacetic acid followed by acylation with 4-methoxybenzoyl chloride in presence of triethylamine afforded compound VIEBAR53 in 52% yield. Application of the same condition using 4-fluorobenzoyl chloride

gave VIEBAR52 in 72% yield. Compounds VIEBAR43 and VIEBAR42 were prepared from **3** using the same procedure used to give VIEBAR53 and VIEBAR52. The yields for VIEBAR43 and VIEBAR42 were 39% and 75%, respectively.

Purity of all six new compounds were evaluated via an analytical high-performance liquid chromatography (HPLC) and showed to be more than 95% (Figure 14).

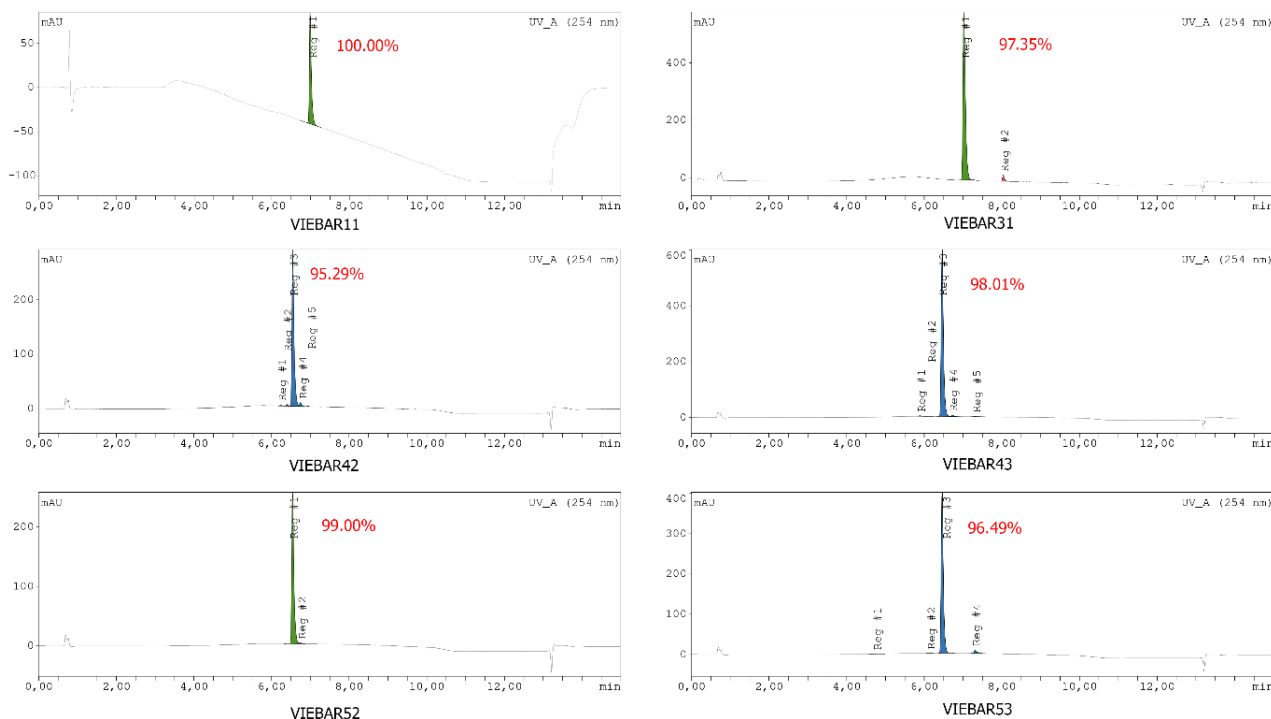


Figure 14. HPLC diagrams for purity evaluations of VIEBAR compounds. (Purity grade shown in red)

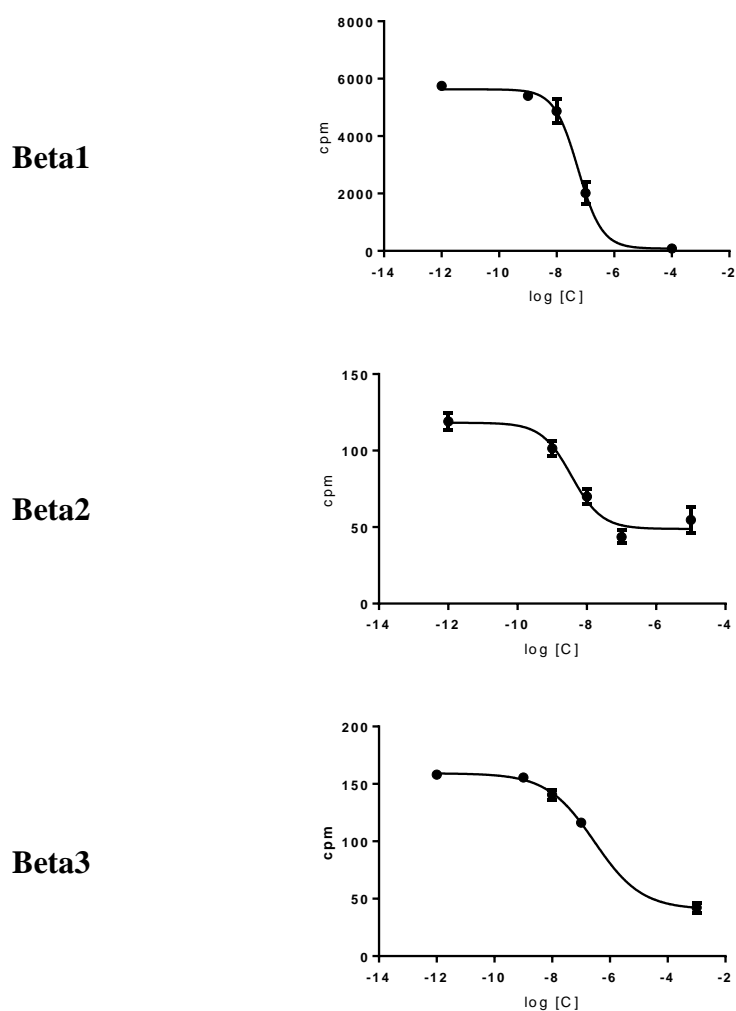
A desirable PET radiotracer for β_2 adrenergic receptors requires high affinity and selectivity for the target (Pike 2009). The affinity of the novel compounds for human adrenergic β_1 , β_2 and β_3 receptors was measured by non-linear regression analysis of membrane bound radioactivity. For this purpose the compounds were analyzed via in vitro radioligand competitive binding assays using [^3H](–)-CGP-12177 for labelling all assays. Employing Cheng and Prusoff's equation (Yung-Chi & Prusoff 1973), the inhibition constants (K_i) for the compounds were calculated.

3.2. Binding data

Binding data for VIEBAR 11:

Representative competitive curves for VIEBAR11 using three beta adrenergic receptor subtypes expressing membranes are given in table 12. For VIEBAR11, mean values of IC₅₀ towards beta1, beta2 and beta3 are 81.75 ± 28 nM (n=3), 3.78 ± 1.5 nM (n=3) and 213 ± 102 nM (n=2), respectively.

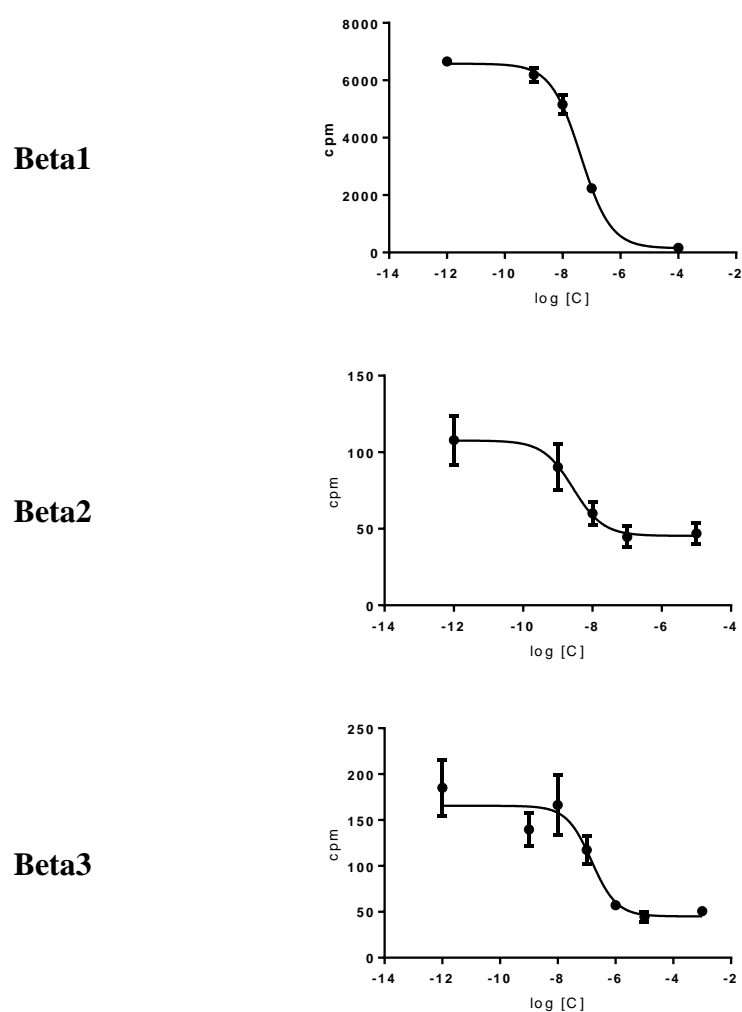
Table 12. Representative competition curves for VIEBAR11 towards beta adrenoceptors



Binding data for VIEBAR 31:

Table13 includes representative competitive curves of VIEBAR31 using beta-1, beta-2 and beta-3 adrenoceptor expressing membranes. For VIEBAR31, mean values of IC₅₀ towards beta1, was calculated 53.5 ± 10.2 nM (n=3) where towards beta2 and beta3 are 3.51 ± 0.93 nM (n=3), and 191 ± 42 nM (n=2), respectively.

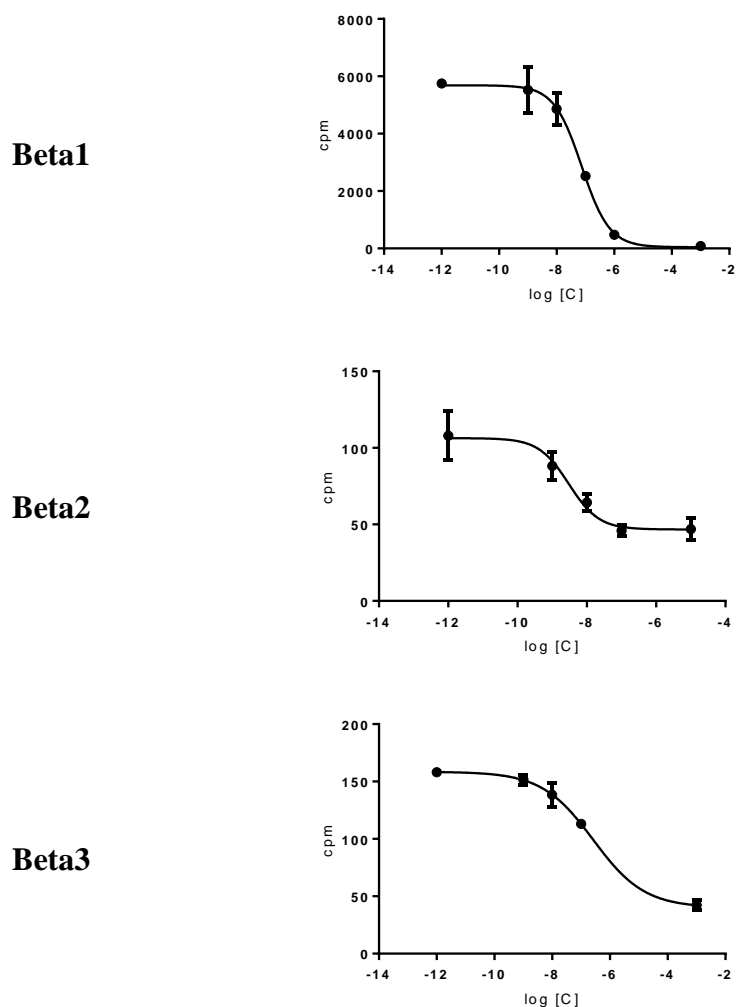
Table 13. Representative competition curves for VIEBAR31 towards beta adrenoceptors



Binding data for VIEBAR 42:

In table 14, representative competition curves of VIEBAR42 resulted from binding assays of beta adrenoceptors are shown. IC₅₀ mean values were calculated 139 ± 53.5 nM (n=3) for beta-1, 3.65 ± 1.7 nM (n=4) for beta-2 and 334 ± 101 nM (n=2) for beta-3 adrenergic receptors.

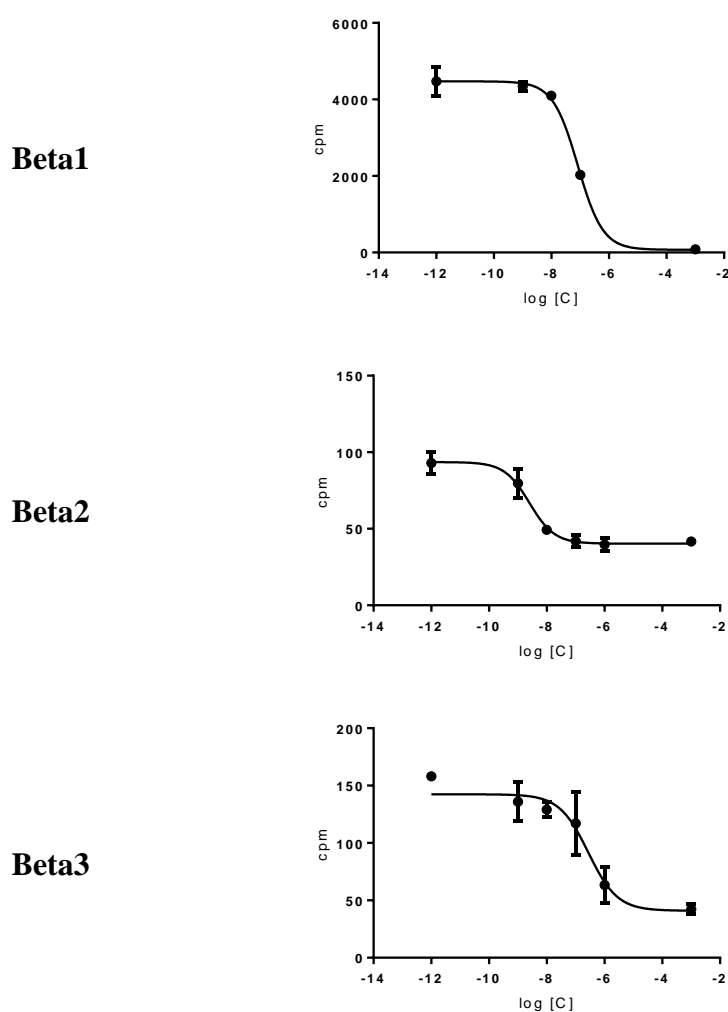
Table 14. Representative competition curves for VIEBAR42 towards beta adrenoceptors



Binding data for VIEBAR 43:

Curves in table 15 refer to competition binding studies of VIEBAR43 towards beta-1, beta-2 and beta-3 adrenoceptors. IC₅₀ mean values for VIEBAR43 were calculated for beta-1 adrenergic receptors 90.5 ± 13.5 nM (n=3), beta-2 adrenergic receptors 2.06 ± 0.6 nM (n=3) and beta-3 adrenergic receptors 199 ± 107 nM (n=2).

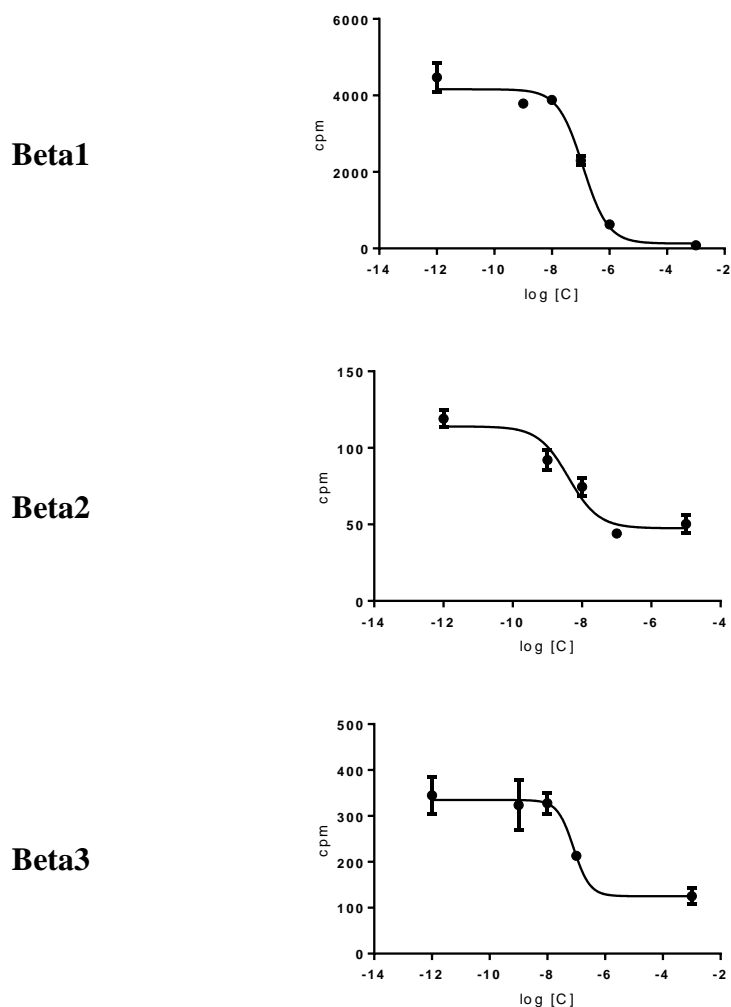
Table 15. Representative competition curves for VIEBAR43 towards beta adrenoceptors



Binding data for VIEBAR 52:

In table 16, three representative competition curves are shown. Mean values of IC₅₀ for VIEBAR52 towards beta-1 adrenoceptor was calculated 119 ± 38.8 nM (n=3) while towards beta-2 and beta-3 adrenoceptors were 12.5 ± 6.1 nM (n=4) and 119 ± 54 nM (n=2), respectively.

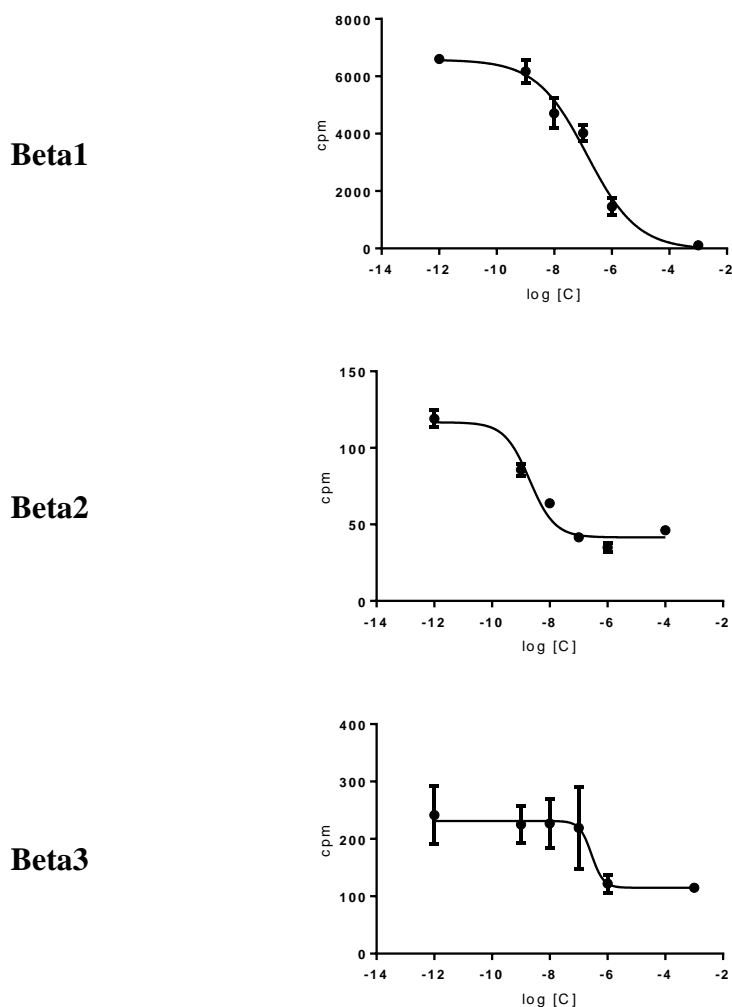
Table 16. Representative competition curves for VIEBAR52 towards beta adrenoceptors



Binding data for VIEBAR 53:

IC₅₀ of VIEBAR53 were calculated towards beta-1 adrenergic receptors 161 ± 30.5 nM (n=3), beta-2 adrenergic receptors 4.32 ± 2.1 nM (n=3) and beta-3 adrenergic receptors 137 ± 17 nM (n=2). Representative competition curves are shown in table 17.

Table 17. Representative competition curves for VIEBAR53 towards beta adrenoceptors



Calculated K_i values are summarized in table 18. As expected, the affinity of all six novel ligands is in a suitable range while approximately all compounds have a sufficient ratio of selectivity against the β_2 -ARs. Moreover, stereoisomers with the absolute configuration (*S*) on the stereogenic carbon had, as expected, a lower K_i value compared to their racemic analogues. The additional nitrogen between carbonyl group and piperidine moiety in **VIEBAR42**, **VIEBAR43**, **VIEBAR52** and **VIEBAR53** had no significant effect on the β_2 -AR binding affinity.

Table 18. Human β_1 , β_2 and β_3 receptor binding affinities of new compounds (K_i values in nM). (a) LogP and polar surface area (PSA) values were calculated via *Mcule.com* online drug discovery platform

Compound	Affinity (K_i)						Selectivity		Permeability ^a	
	β_1	N	β_2	N	β_3	N	β_2/β_1	β_2/β_3	PSA	LogP
VIEBAR11	24.2 ± 8.3	3	0.55 ± 0.2	3	211 ± 101	2	44	384	65.6	4.73
VIEBAR31	15.8 ± 3	3	0.51 ± 0.1	3	189 ± 42	3	31	372	65.6	4.73
VIEBAR42	41.1 ± 15.8	3	0.53 ± 0.2	4	330 ± 100	2	77.6	622	77.6	4.42
VIEBAR43	26.8 ± 4	3	0.3 ± 0.09	3	197 ± 106	2	89.2	656	86.8	4.29
VIEBAR52	35.1 ± 11.5	3	1.44 ± 0.9	4	118 ± 53	2	24.4	82	77.6	4.42
VIEBAR53	47.7 ± 9	3	0.63 ± 0.3	3	135 ± 17	2	75.6	214	86.8	4.29

VIEBAR43 and VIEBAR53, which have a methoxy group connected to the benzoyl group showed a lower K_i value in comparison with VIEBAR42 and VIEBAR52, the analogous fluorine containing compounds. It should be noted that there is an inverse relation between VIEBAR11 and (1-(3-((9H-carbazol-4-yl)oxy)-2-hydroxypropyl)piperidin-4-yl)(4-methoxyphenyl)methanone with no additional amine group as already reported (Tasler 2010). As predicted, the (*S*)-enantiomers VIEBAR43 and VIEBAR42 demonstrate a better binding affinity or selectivity than the racemic compounds VIEBAR53 and VIEBAR52. Although the compounds VIEBAR43 and VIEBAR42 with higher selectivity are better candidates for the further evaluations, based on the previously mentioned rules for an appropriate compound passing the BBB, VIEBAR11 and VIEBAR31 would be the more appropriate choices for a CNS β_2 -AR PET ligand considering their lower calculated PSA.

Although the calculated LogP of compounds are in the desirable range, we estimate that the compounds are still highly lipophilic. Lowest lipophilicity is predicted for VIEBAR43 and VIEBAR53 which both have high PSAs. In contrast, VIEBAR11 and VIEBAR31 with the lowest PSAs are probably high lipophilic. Considering K_i values, selectivity ratios and LogP and PSA data, all compounds are suitable ligands for beta-2 adrenergic receptors although structural modifications are needed for development of these novel ligands.

Radioligand binding assay was established based on the protocols recommended by Perkin Elmer. Nevertheless, we had to change several parameters of assays according to the conditions. Temperature influences both equilibrium constants and rates of reaction. Incubation in lower temperatures requires longer time to allow equilibration to be achieved. We kept the incubation temperature constant on 27 °C. Based on the trial and error experiments, the incubation time for beta-2 adrenoceptor binding assay was increased to ninety minutes. Additionally, the incubation time for beta-3 adrenoceptor binding assay was thirty minutes. Furthermore, the glass fiber filter type used for beta-2 adrenoceptor binding assay was GF/C whose pore size is 1.2 µm. Due to the low counts achieved from the beta-2 binding experiments, we preferred to use GF/B filters with the pore size of 1 µm for beta-1 and beta-3 experiments.

In the case of membranes expressing beta-2 and beta-3 adrenergic receptors, the protein concentrations were 1 µg/µL and 2 µg/µL, respectively. We speculate that the low protein concentration caused the few counts for beta-2 and beta-3 binding assays. The low counts results the short interval between specific and non-specific binding that influences the precision of the whole experiments. Therefore, the calculated errors in some cases are considerable. However, the competition curves of beta-2 adrenergic receptor were acceptable. Initial experiments with their results are not referred here, did not represent desirable results for beta-2 adrenoceptors binding study. The problem was solved by exchanging the membrane lot.

Conclusions and future insights

In summary, all six synthesized compounds displayed a receptor binding affinity in the desired range. The selectivity against β_1 -ARs was not sufficiently high for VIEBAR52 and VIEBAR31 to be used as selective ligands. The K_i values confirmed that the enantiomerically enriched compounds with an (S)-configuration at the stereogenic carbon showed a higher receptor binding affinity. From our results, we expect that VIEBAR11 and VIEBAR31 will play a better role as cortical and cerebral β_2 -AR PET-ligands in comparison to the other compounds, whereas VIEBAR42 and VIEBAR43 will be better choices for cardiac imaging.

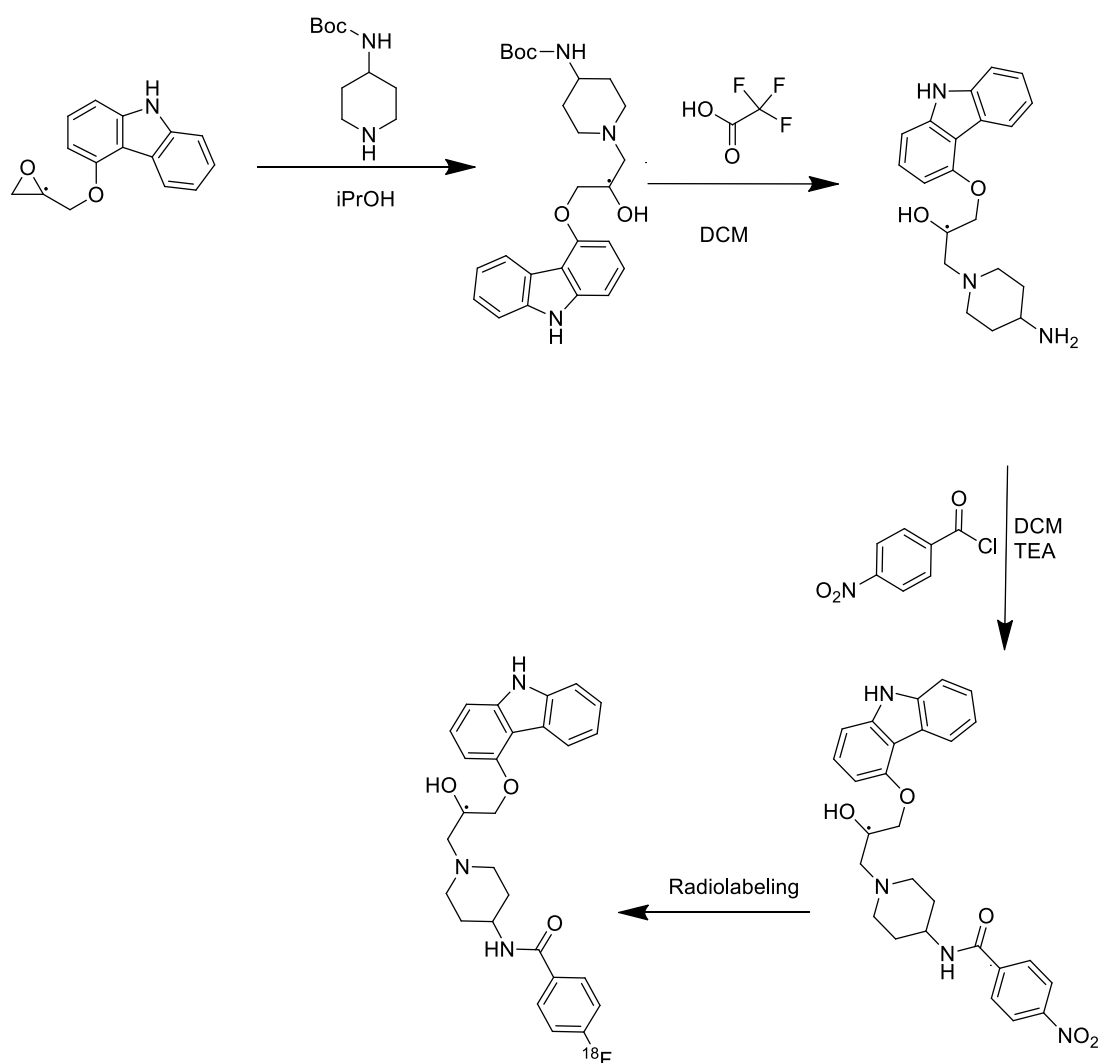


Figure 15. Proposed synthesis strategy for radiosynthesis of $[^{18}\text{F}]$ VIEBAR42 and $[^{18}\text{F}]$ VIEBAR52

According to the binding data, further preclinical tests can determine the potency of VIEBAR compounds towards beta-2 adrenoceptors. Evaluation of experimental LogP, blood brain barrier permeability, plasma protein binding and whether the compounds are substrates to efflux-transporters, respectively, is necessary and can be performed using these compounds in their non-radioactive form.

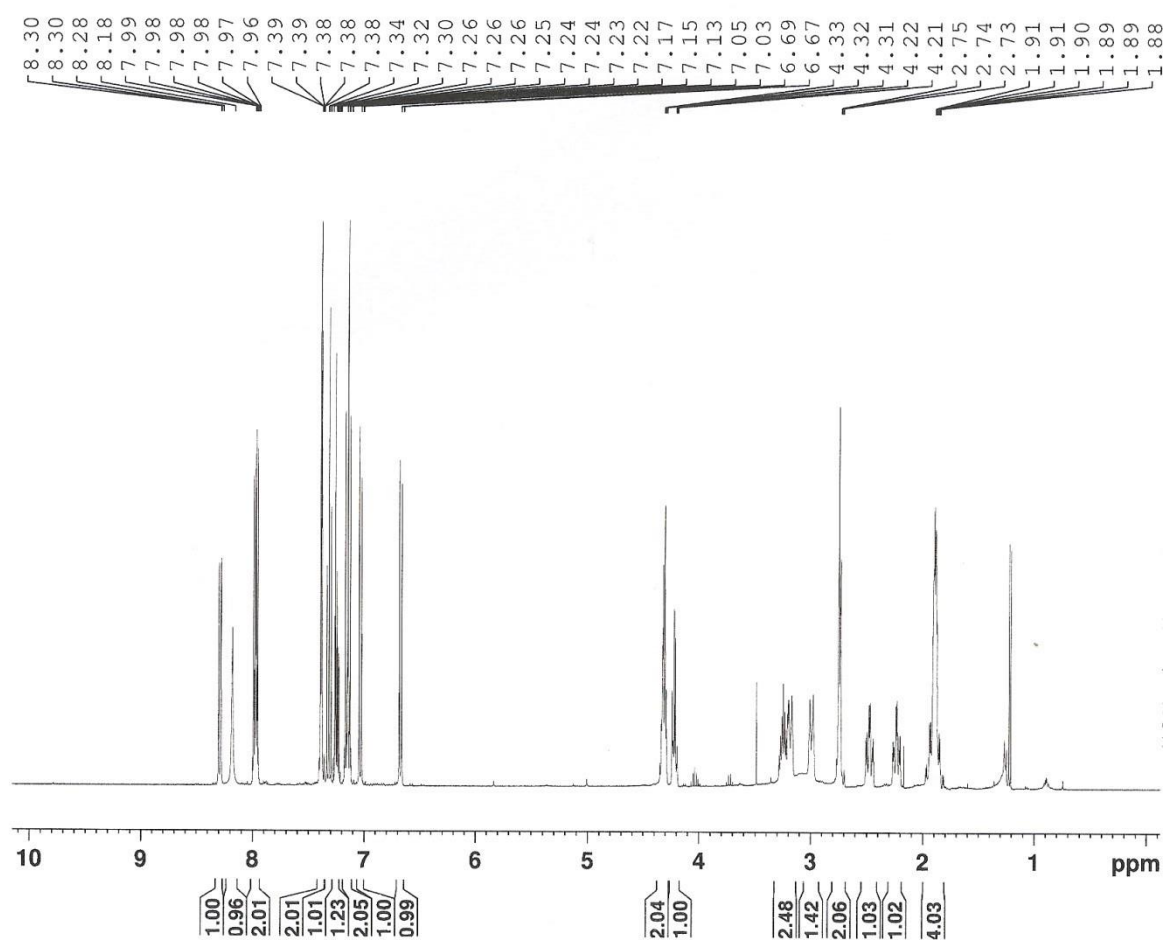
Among the compounds, VIEBAR42 had the best overall properties with respect to its receptor binding affinity and selectivity. For radiolabeling of VIEBAR42 (and VIEBAR52), a strategy is suggested in Figure 15.

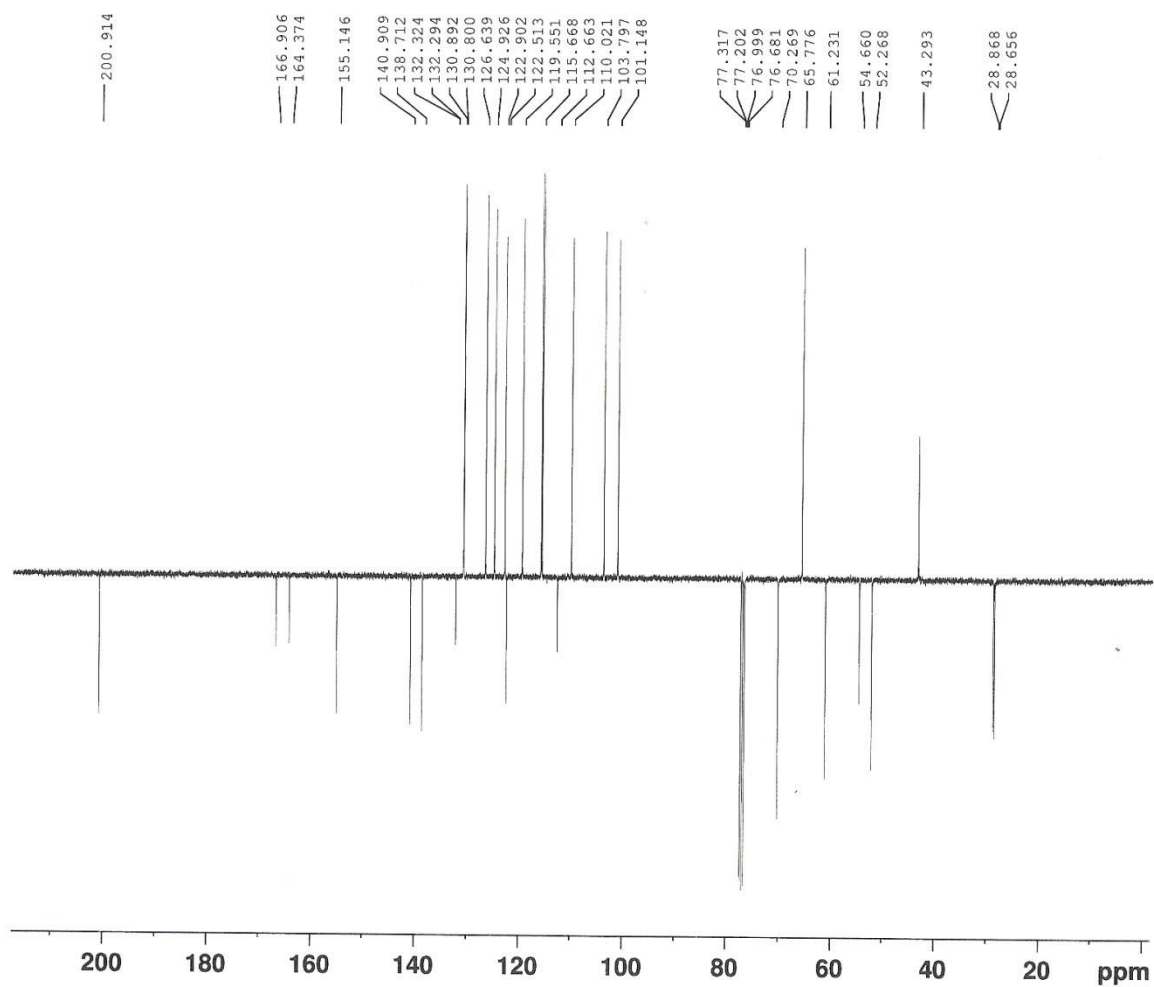
Appendices

Spectral data of novel compounds

VIEBAR11 ¹H-NMR

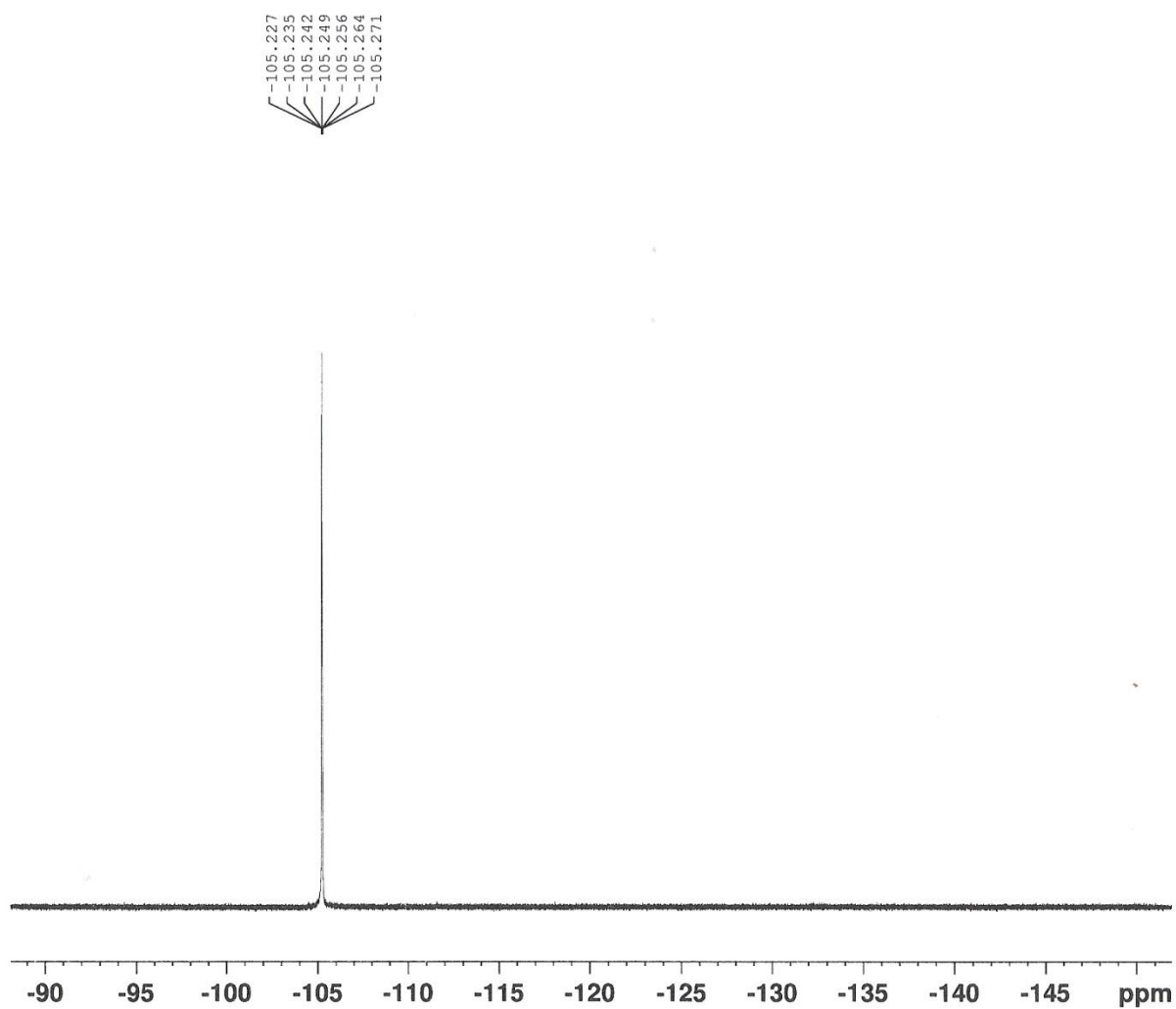
VIEBAR11/ CDCl₃

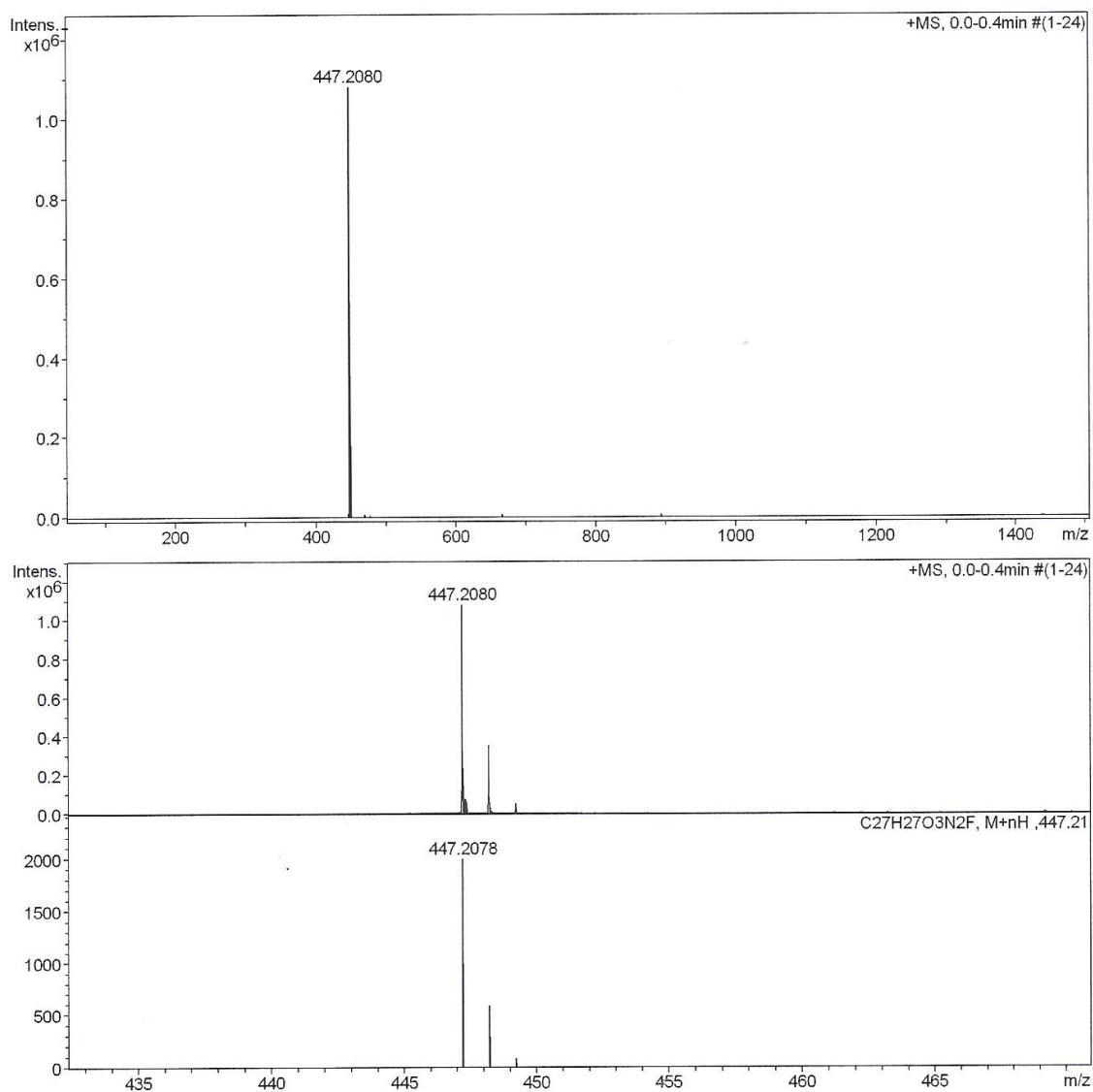


VIEBAR11
¹³C-NMRVIEBAR11/ CDCl₃

VIEBAR11
F19-NMR

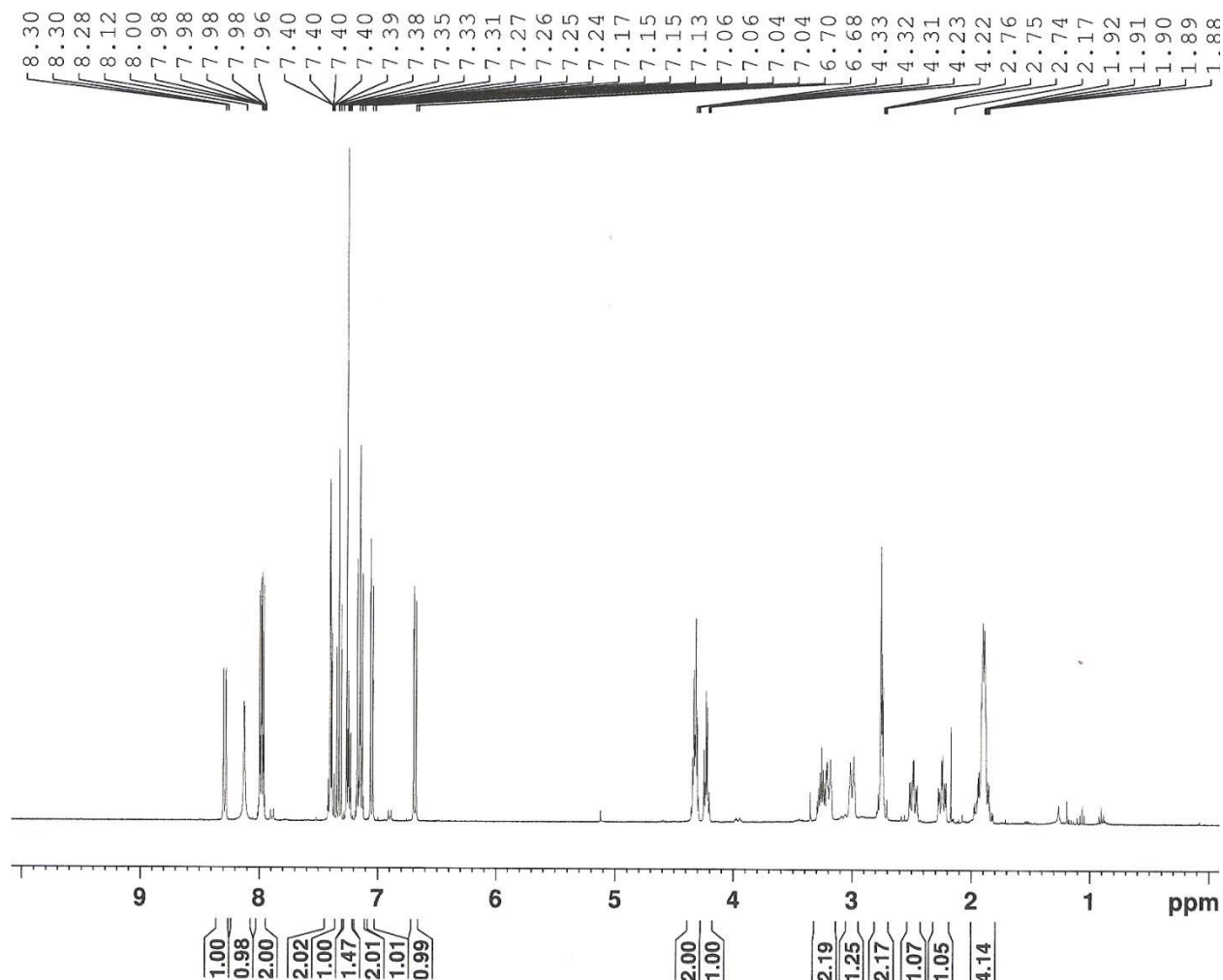
VIEBAR11/ CDC13

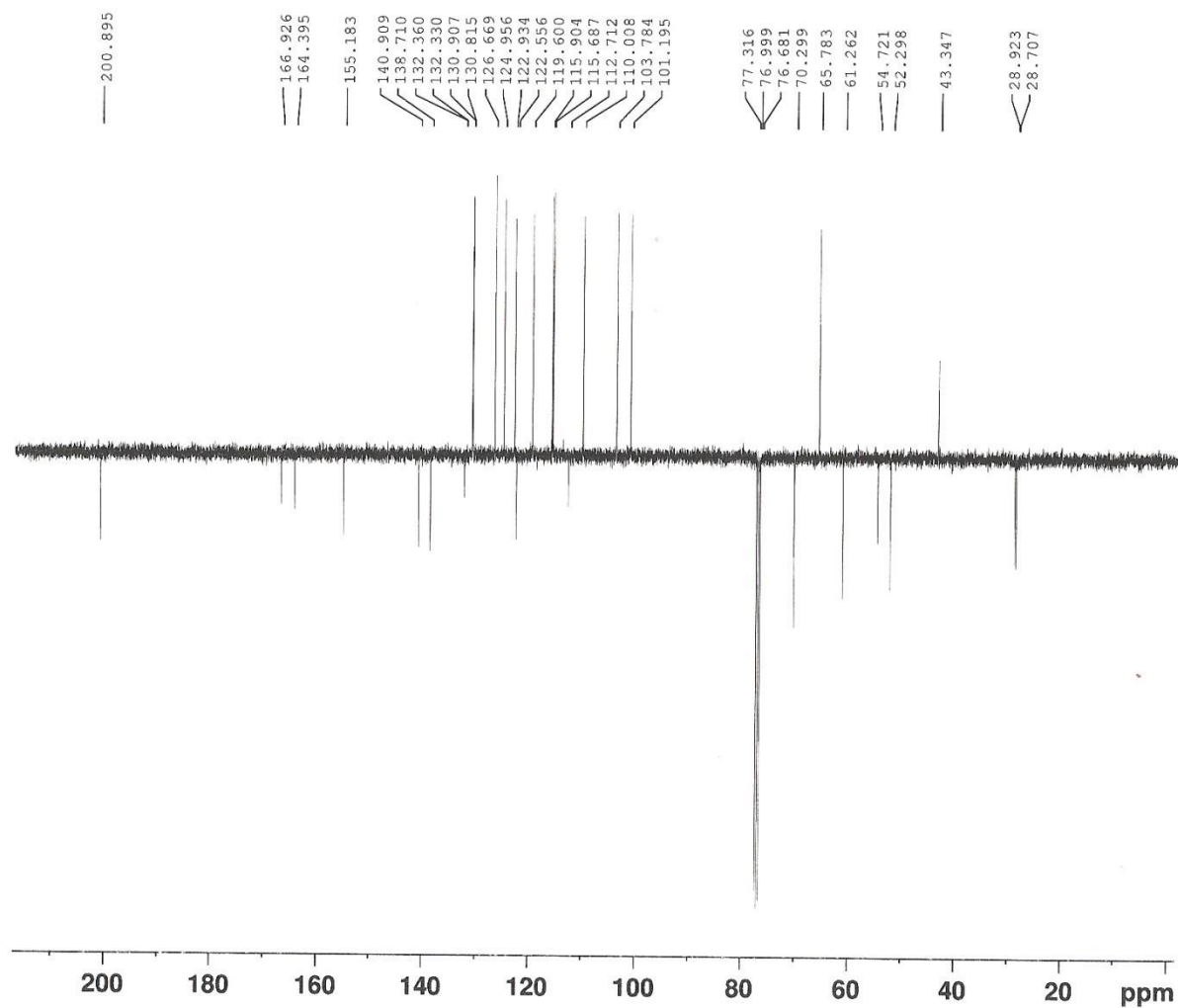


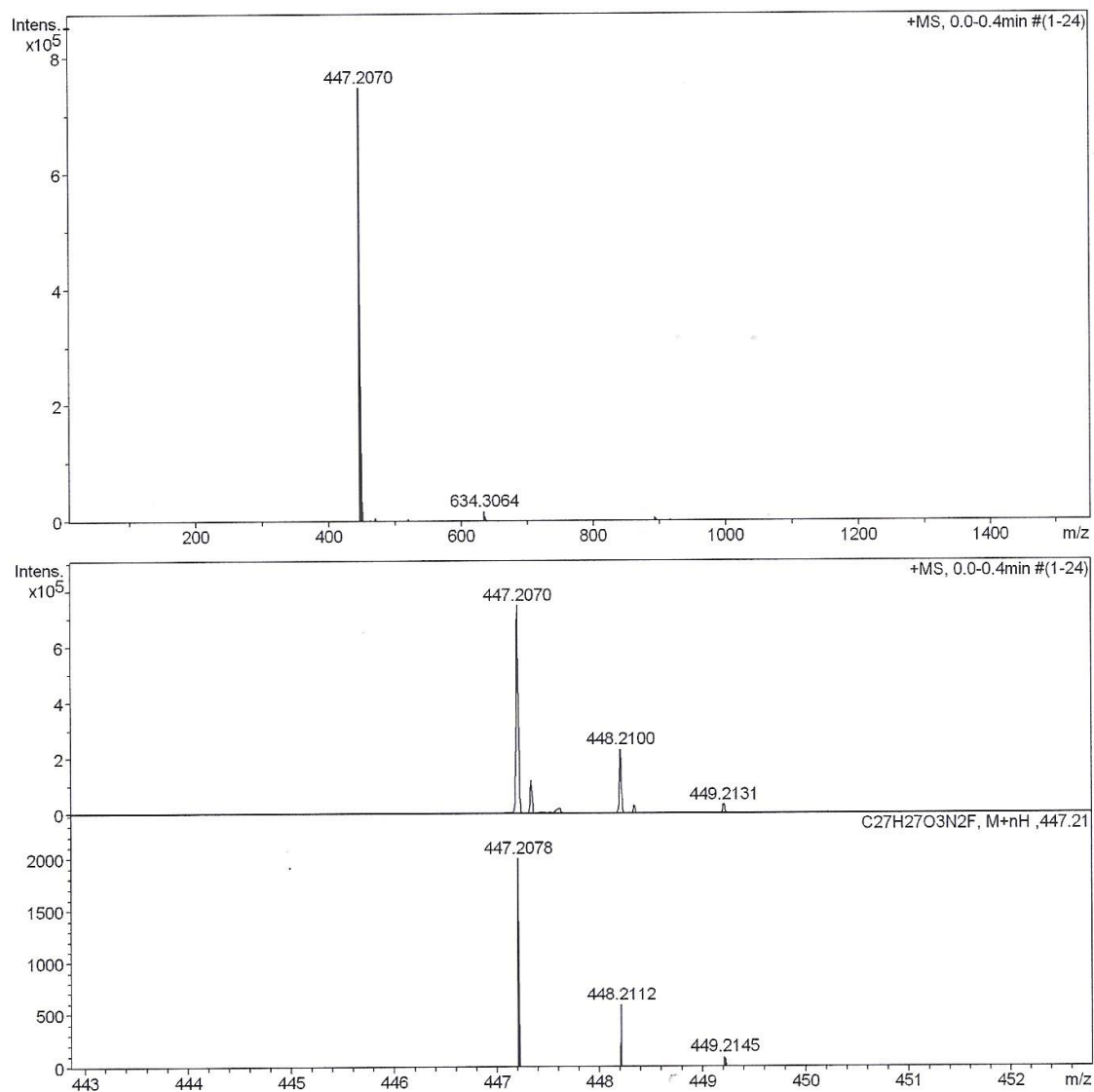
VIEBAR11
ESI-HRMS

VIEBAR31
¹H-NMR

VIEBAR31/ CDC13

similar to VIEBAR 11

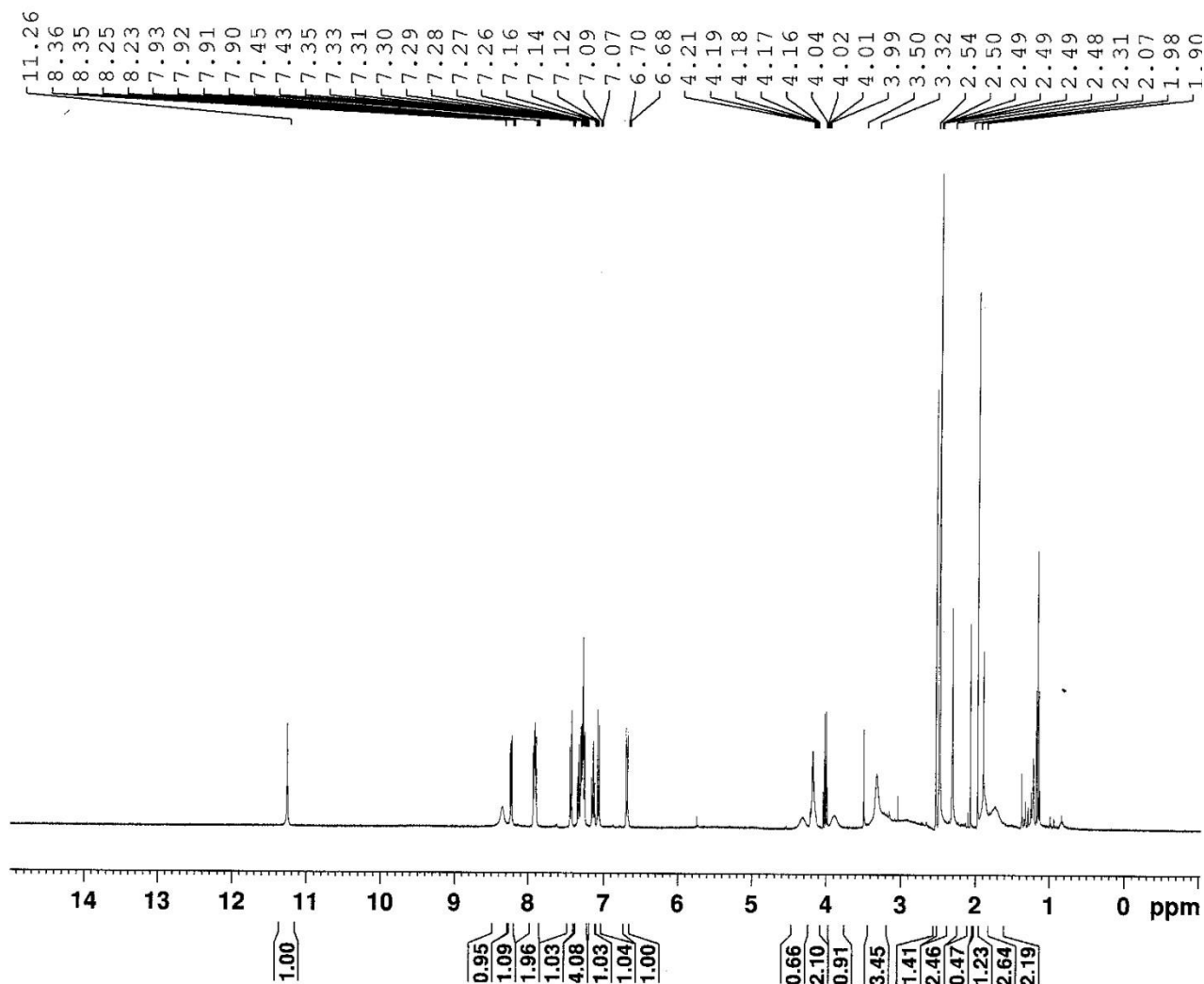
VIEBAR31
¹³C-NMRVIEBAR31/ CDCl₃

VIEBAR31
ESI-HRMS

VIEBAR42**¹H-NMR**

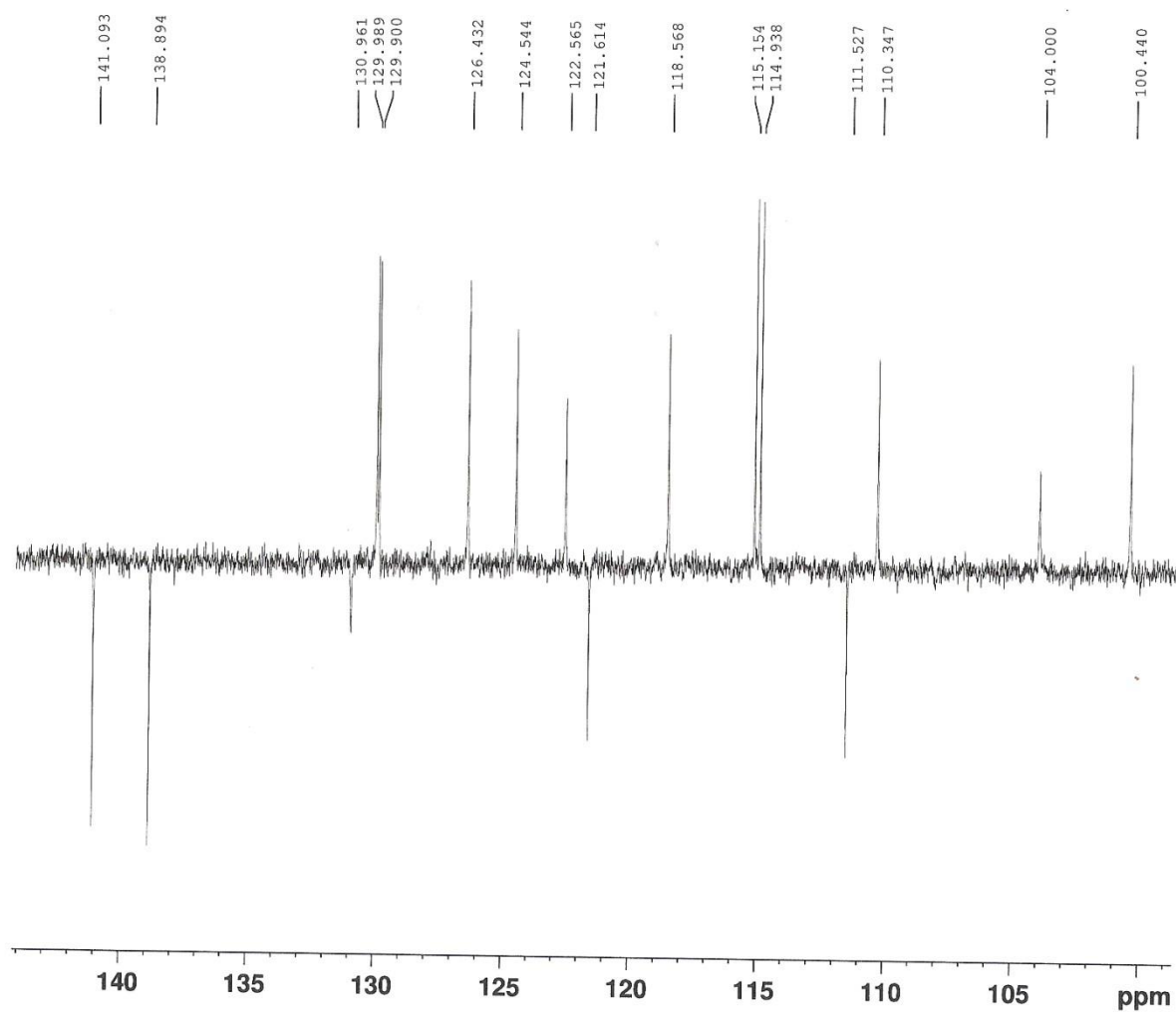
VIEBAR42/ DMSO

Jinfa VIEBAR52



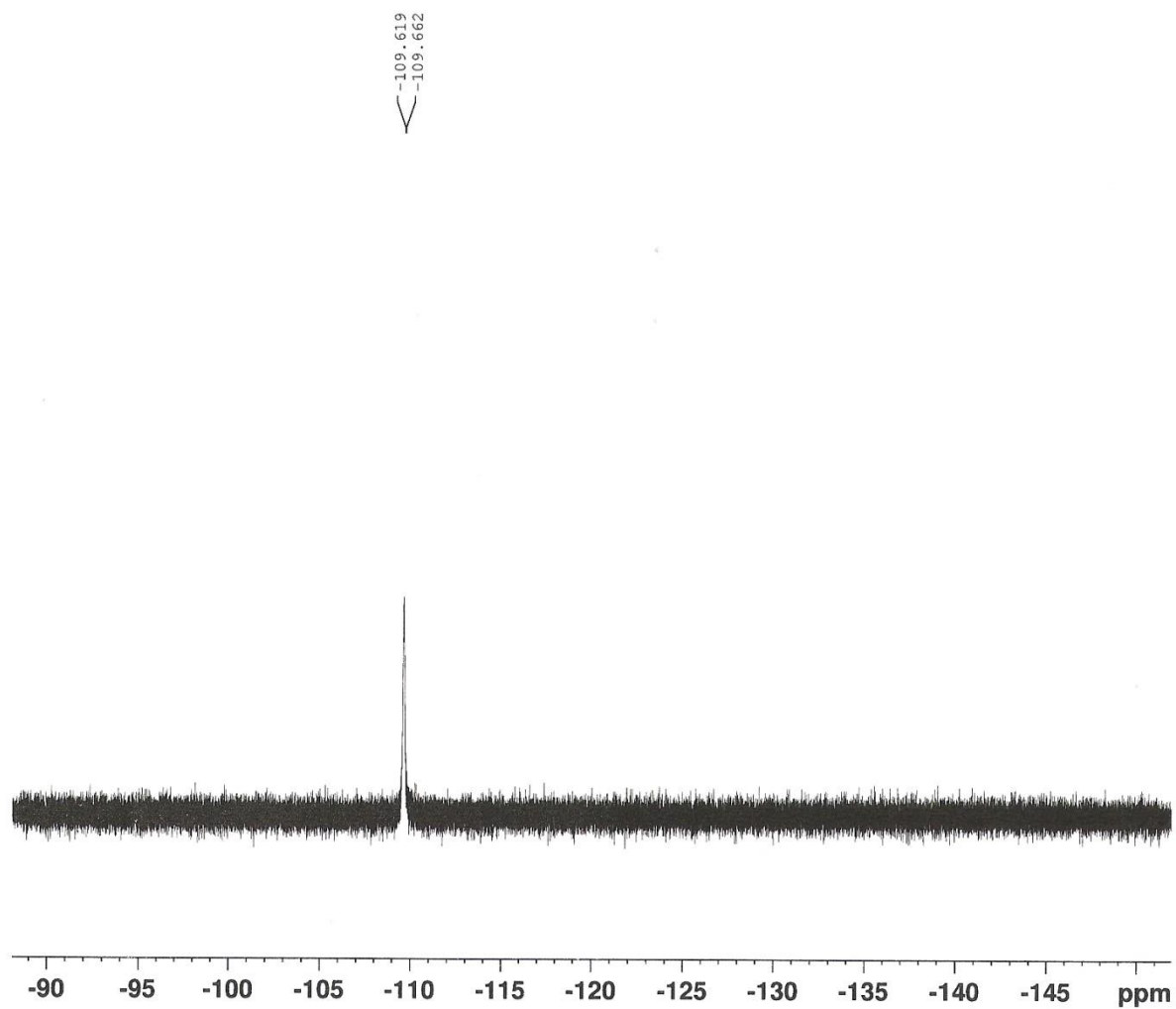
VIEBAR42
¹³C-NMR

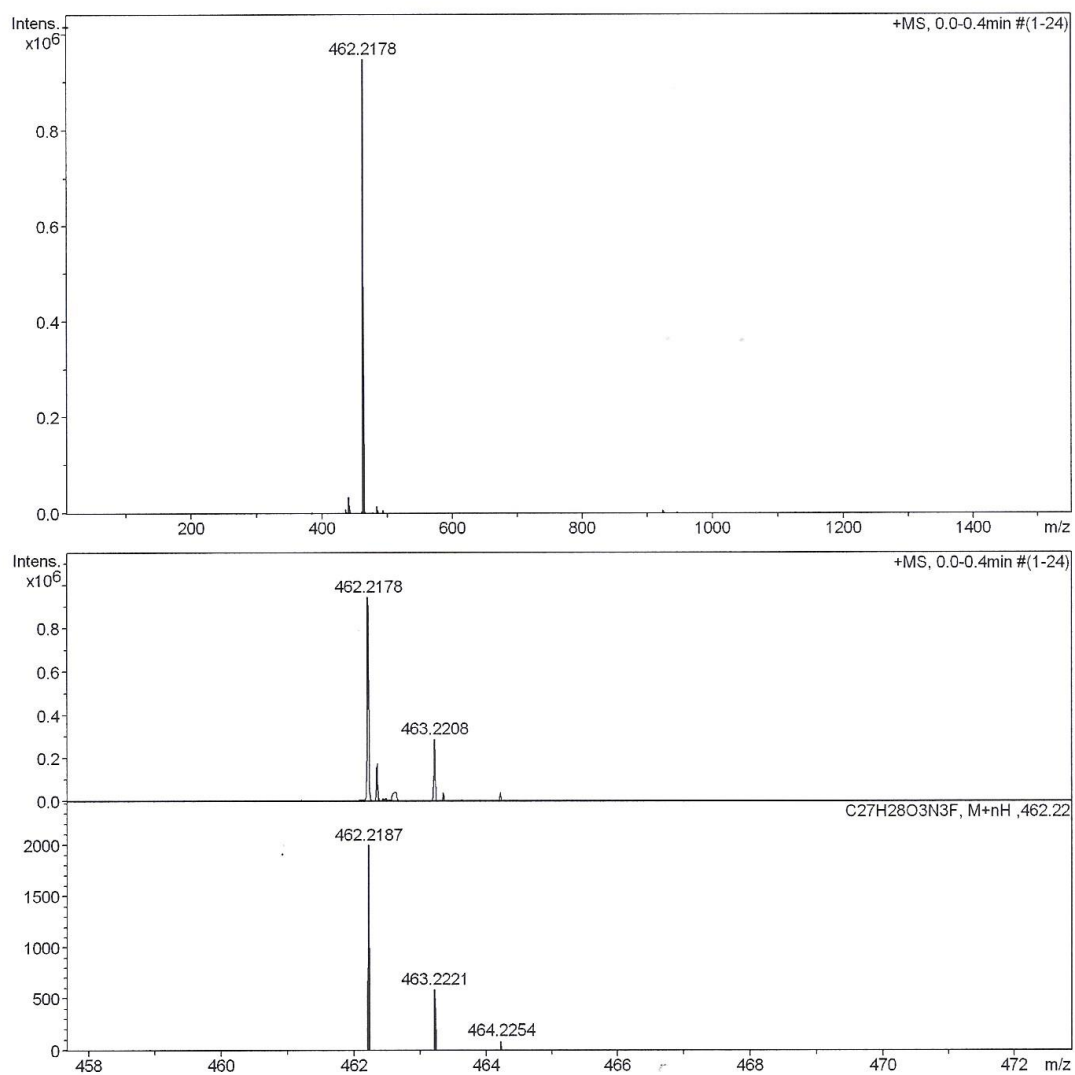
VIEBAR42/ DMSO



VIEBAR42**¹⁹F-NMR**

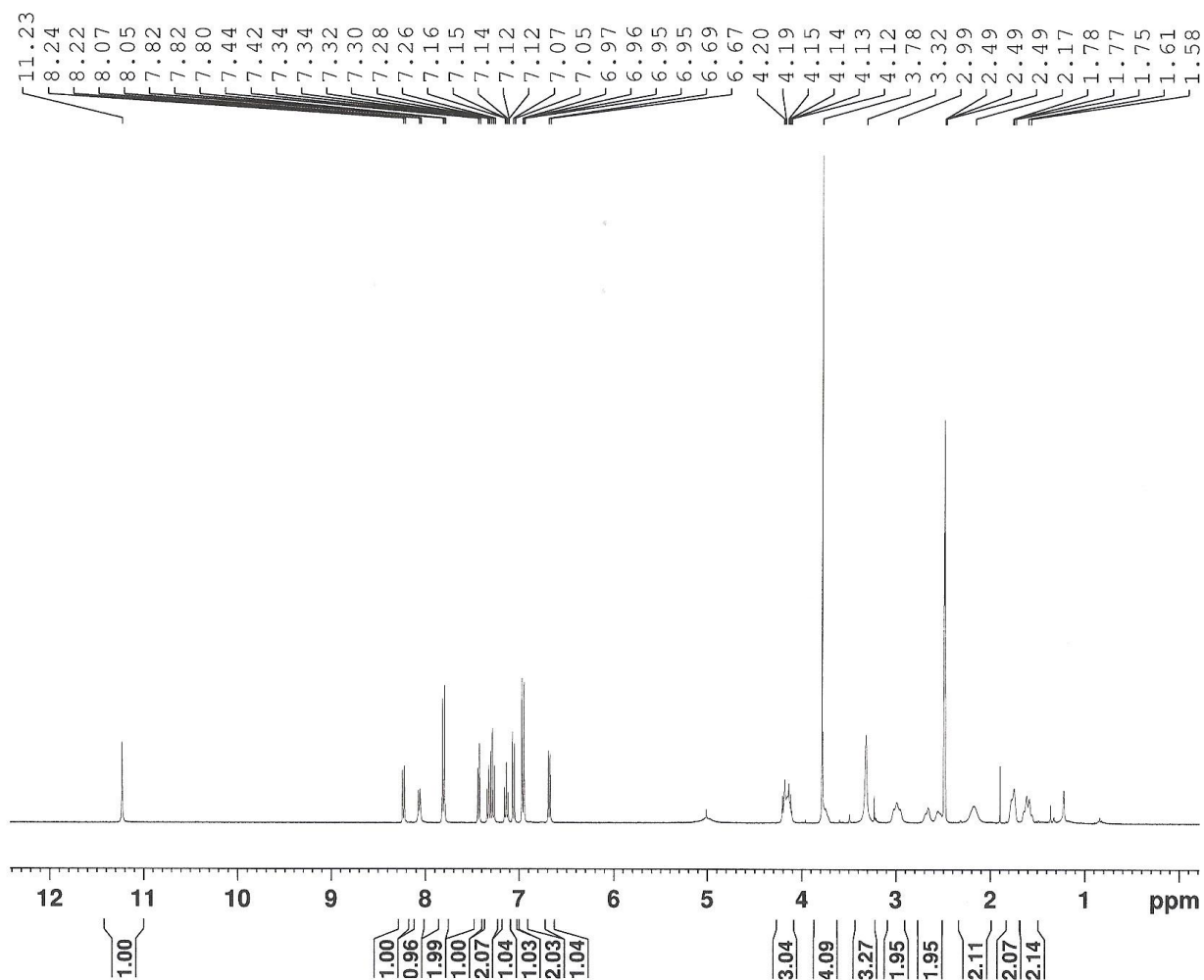
VIEBAR42/ DMSO



VIEBAR42
ESI-HRMS

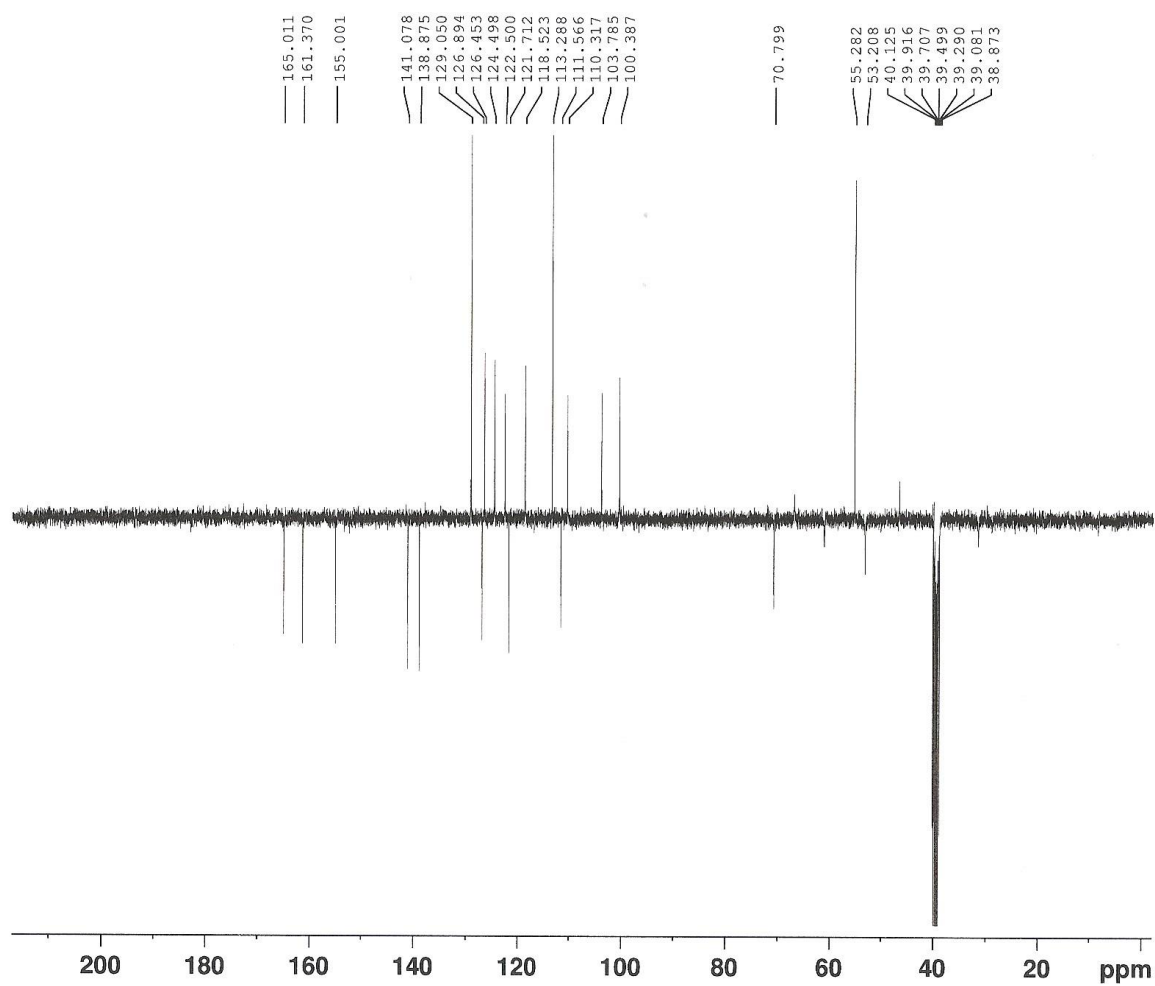
VIEBAR43
¹H-NMR

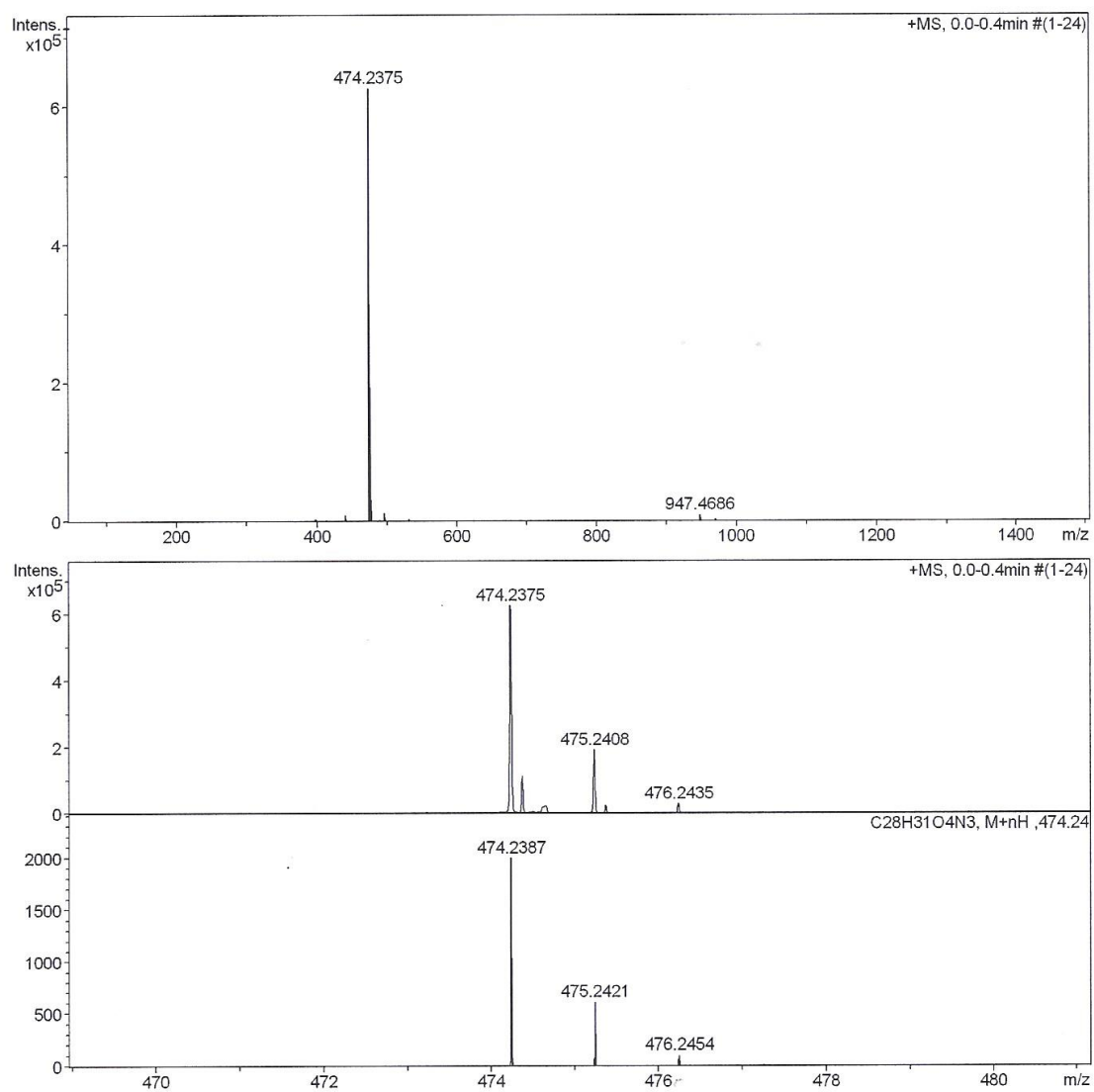
VIEBAR43/ DMSO

Similar to VIEBAR53

VIEBAR43
¹³C-NMR

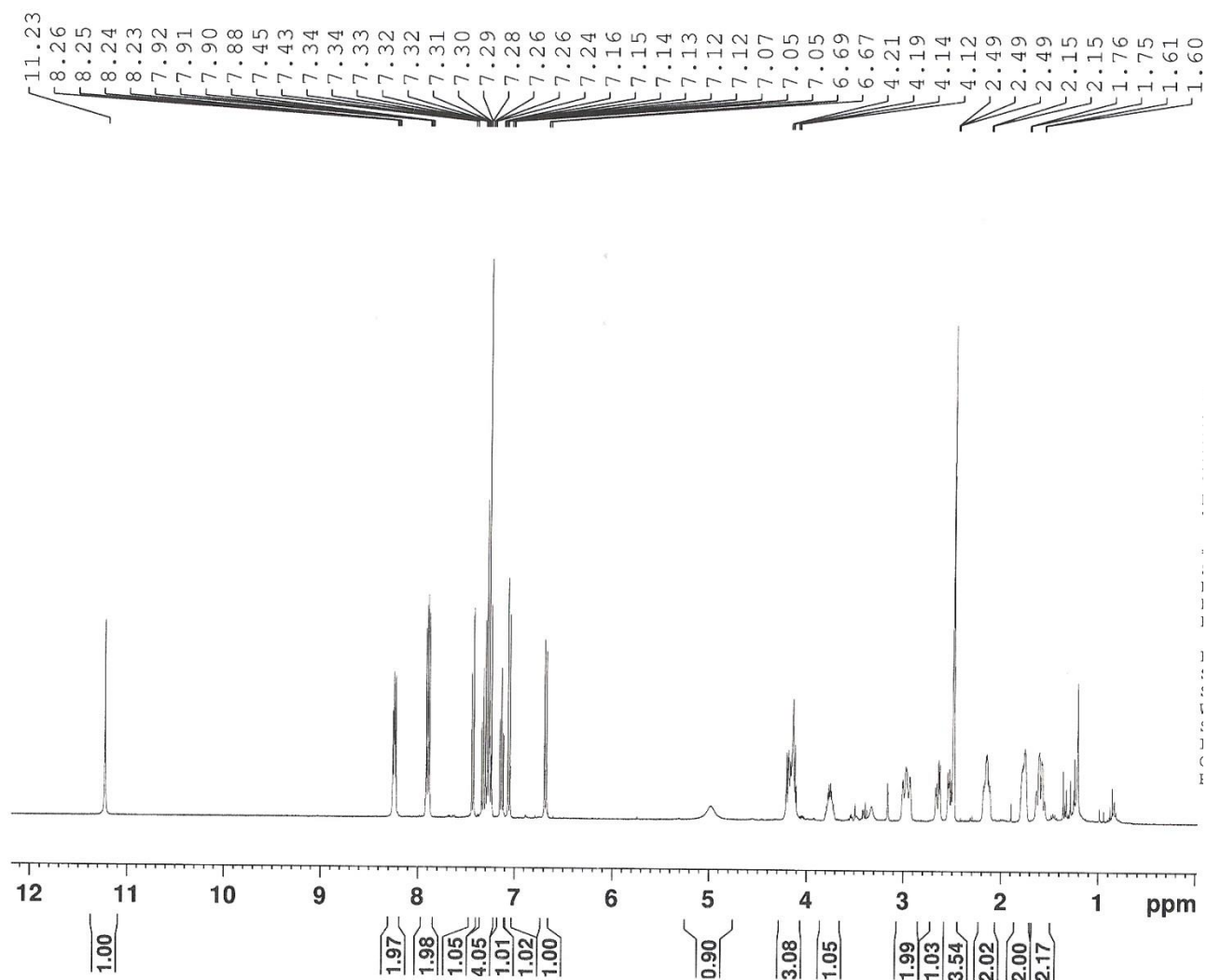
VIEBAR43/ DMSO



VIEBAR43
ESI-HRMS

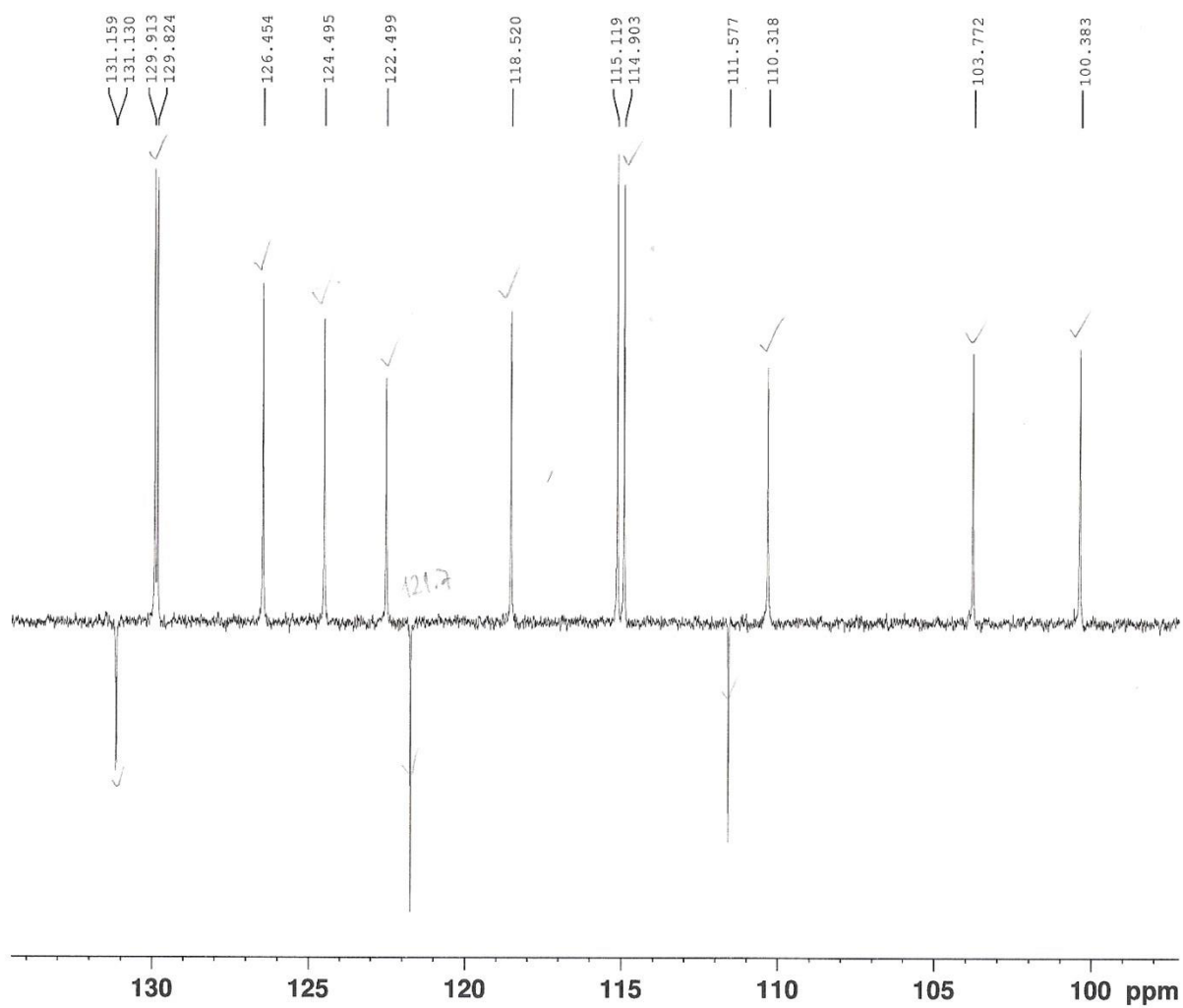
VIEBAR52**¹H-NMR**

VIEBAR52/ DMSO



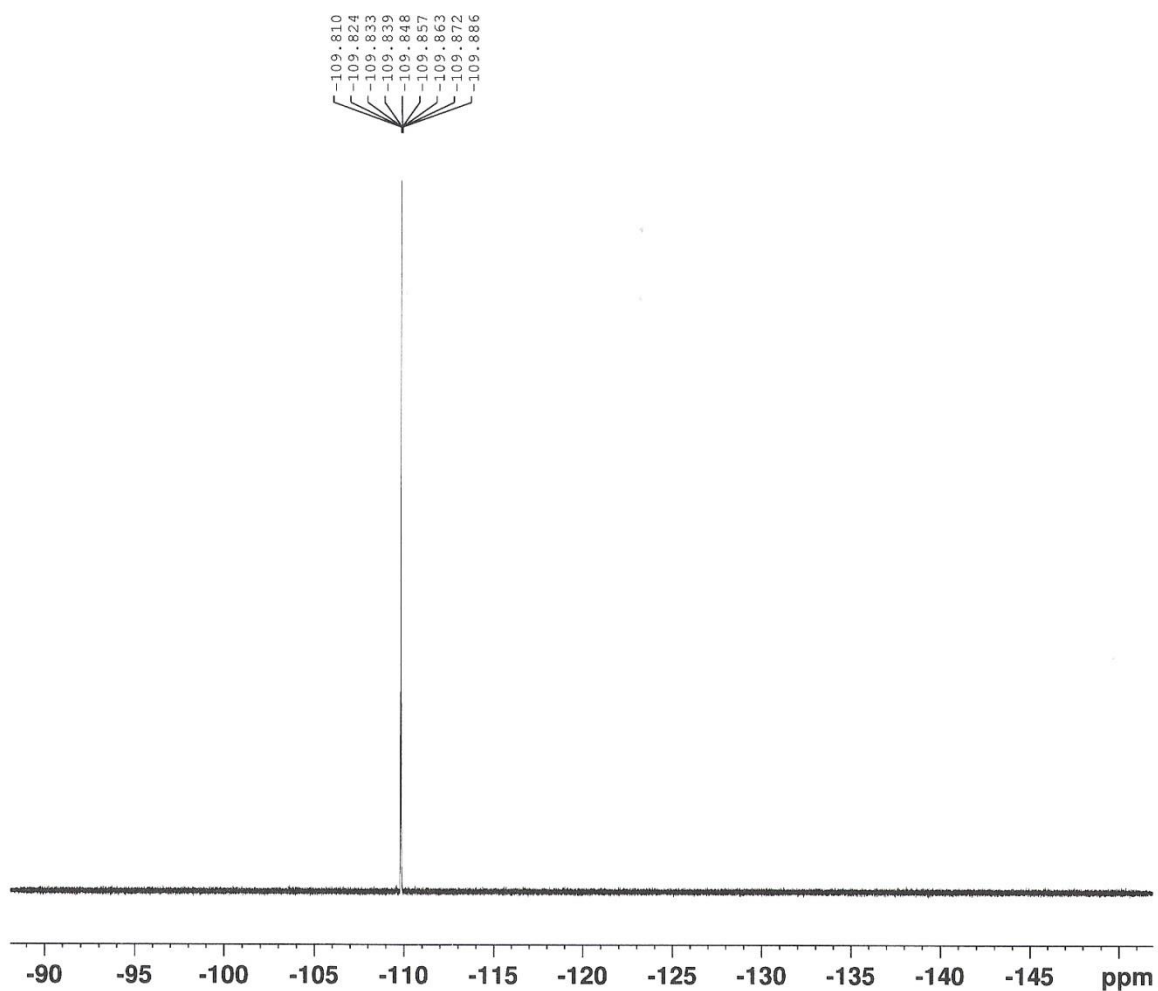
VIEBAR52
¹³C-NMR

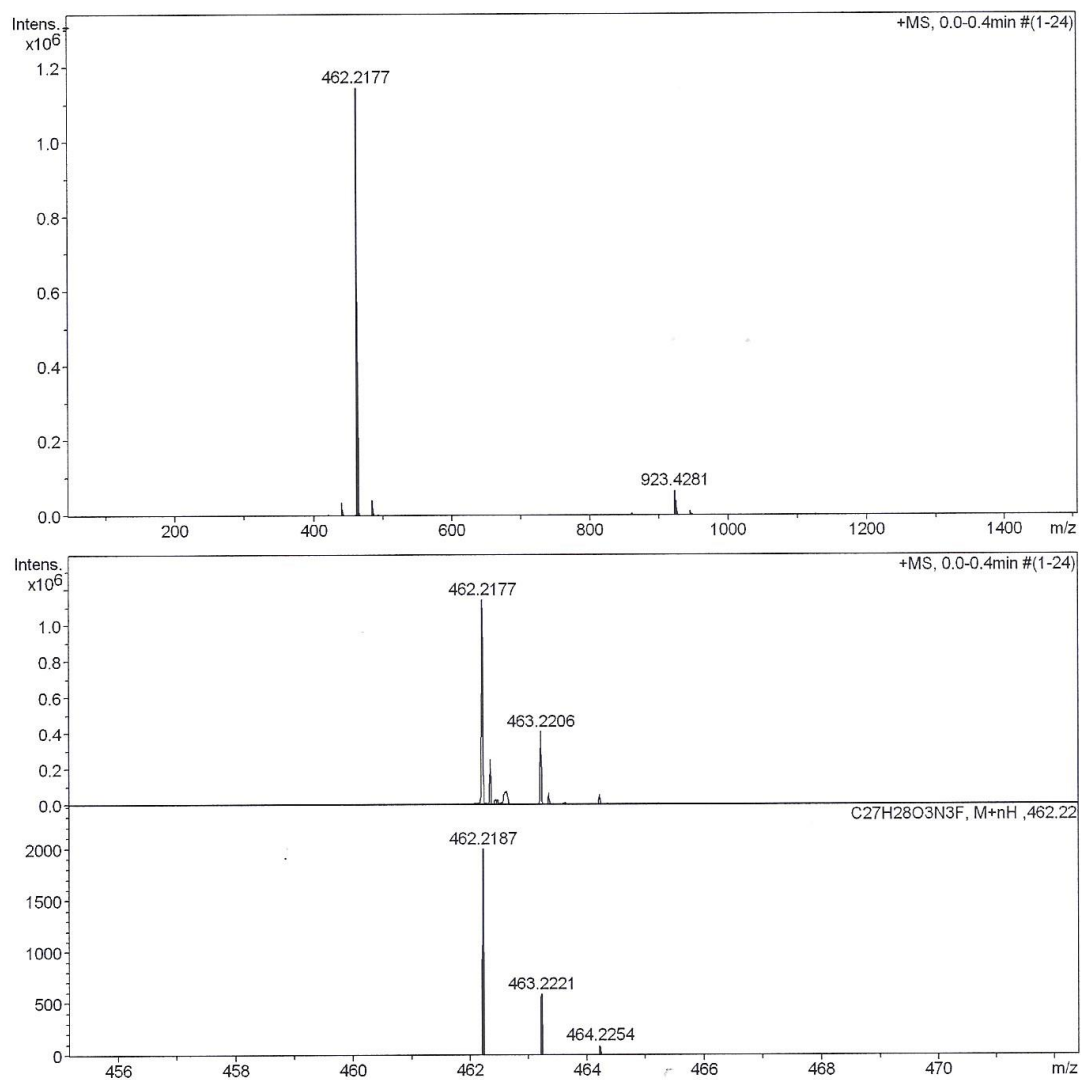
VIEBAR52/ DMSO



VIEBAR52
¹⁹F-NMR

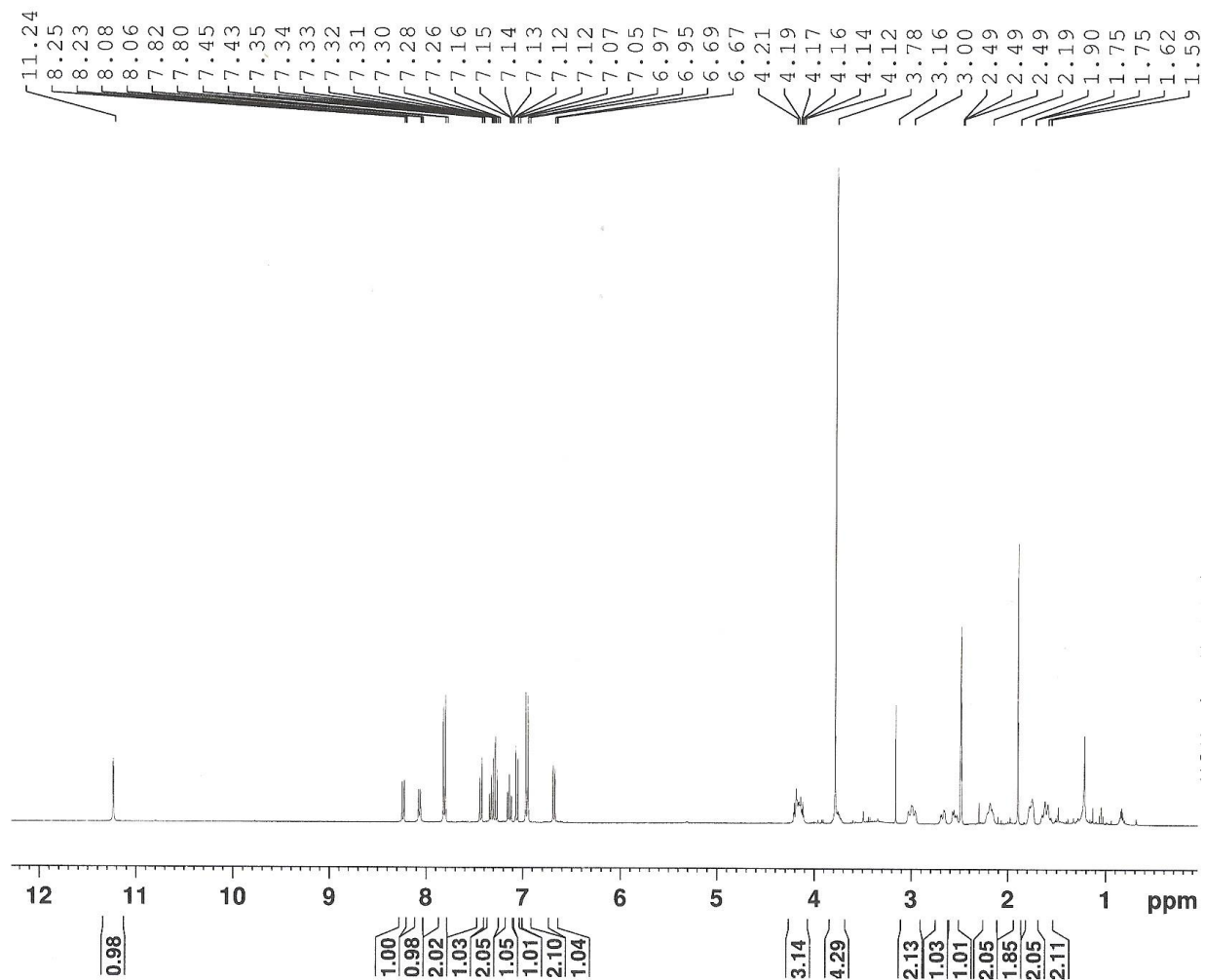
VIEBAR52/ DMSO



VIEBAR52
ESI-HRMS

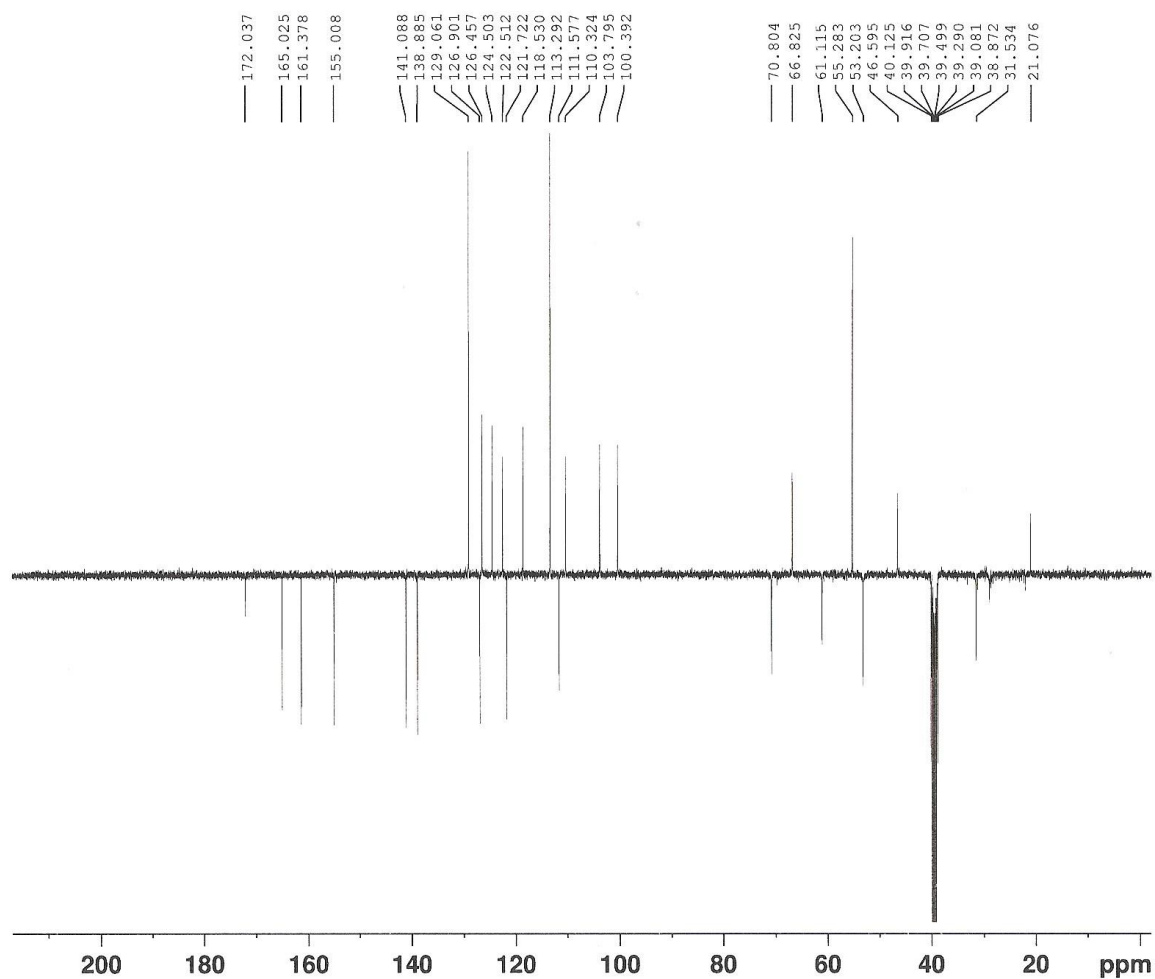
VIEBAR53**¹H-NMR**

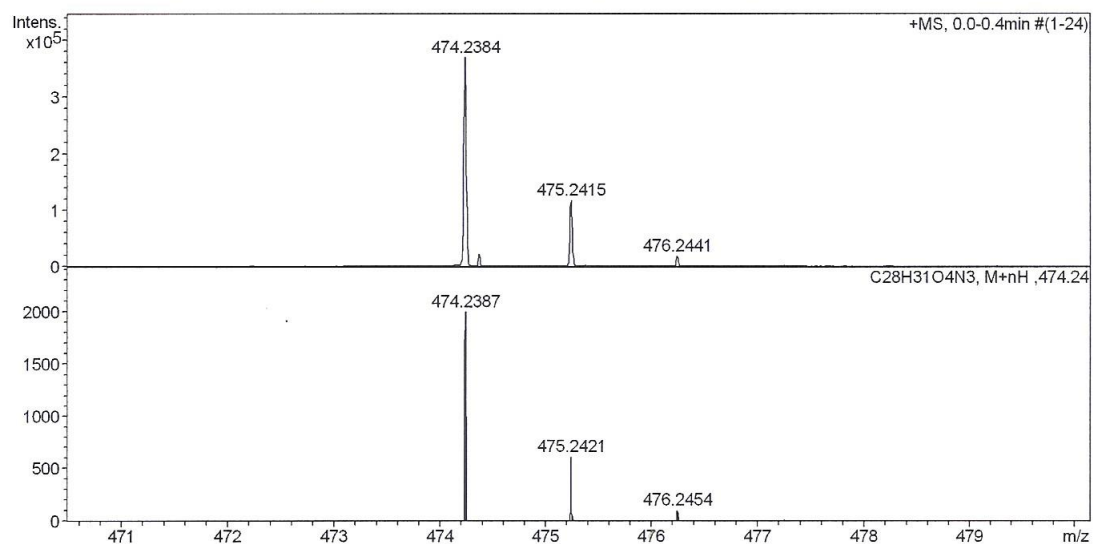
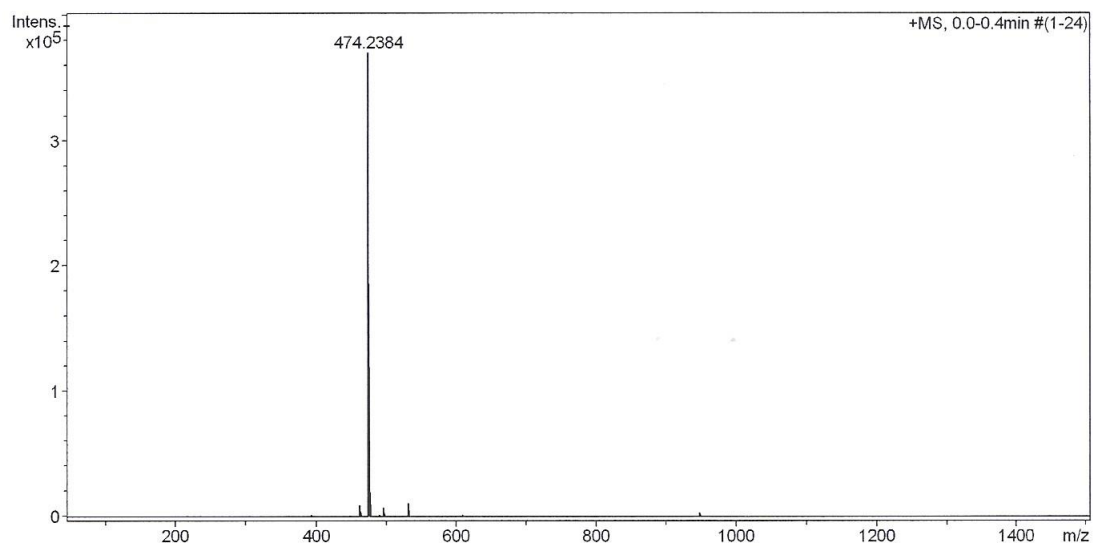
VIEBAR53/ DMSO



VIEBAR53
¹³C-NMR

VIEBAR53/ DMSO



VIEBAR53
ESI-HRMS

Technical data sheet of Perkin Elmer products

TECHNICAL DATA SHEET

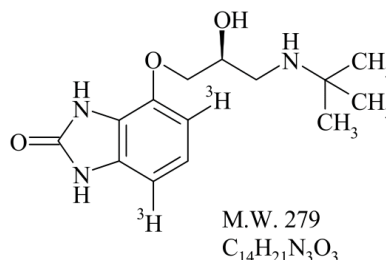
³H

Caution: For Laboratory Use. A product for research purposes only.

(-)-CGP-12177, [5,7-³H]-**Product Number: NET1061**

LOT SPECIFIC INFORMATION

Lot Number:	2094441
Specific Activity:	34.7 Ci/mmol
	1283.9 GBq/mmol
Production Date:	2 December 2015



PACKAGING: 1.0 mCi/ml (37 MBq/ml) in ethanol, under nitrogen, in a silanized vial. Shipped in dry ice.

STABILITY AND STORAGE RECOMMENDATIONS: The stability of (-)-CGP-12177, [5,7-³H]- is currently being evaluated. Initial studies indicate that when (-)-CGP-12177, [5,7-³H]- is stored at -20°C in its original solvent and at its original concentration, the rate of decomposition is initially 3% for 12 months from date of purification. Stability is nonlinear and not correlated to isotope half-life. Lot to lot variation may occur.

SPECIFIC ACTIVITY RANGE: 30-60 Ci/mmol (1110-2220 GBq/mmol)

RADIOCHEMICAL PURITY: This product was initially found to be greater than 97% when determined by the following method. The rate of decomposition can accelerate. It is advisable to check purity prior to use:

High pressure liquid chromatography on Zorbax ODS column using the following mobile phase:
1% triethylamine acetate, pH4 : acetonitrile, (85:15).

QUALITY CONTROL: The radiochemical purity of (-)-CGP-12177, [5,7-³H]- is checked at appropriate intervals using the listed chromatography method.

TRITIUM NMR: The position of label and relative distribution have been investigated by tritium NMR.

REFERENCES:

1. M. Staehelin, P. Simons, K. Jaeggi, and N. Wigger, *J. Biol. Chem.*, **258**, 3496 (1983).
2. M. Riva and I. Crease, *Mol. Pharmacol.*, **36**, 201 (1989).

HAZARD INFORMATION: **WARNING:** This product contains a chemical known to the state of California to cause cancer.

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human Adrenergic β_1 Receptor
Product No.: ES-033-M400UA
Lot No.: 1851571
Material Provided
Membranes: 1 x 400 units / 400 μ L frozen aliquot

Product Information

Cellular Background: CHO-K1
GenBank Accession Number: J03019
Unit Size: 10 μ g protein / unit
Storage Buffer: 50 mM Tris-HCL (pH 7.4), 0.5mM EDTA, 10mM $MgCl_2$, 10% sucrose.
Storage Conditions: Store at -80°C. **Freeze-thaw is not recommended** as it can affect product performance and homogeneity. In order to minimize negative impact of freeze-thawing, flash freeze in liquid nitrogen for 30 seconds prior to transferring to -80°C.
Stability: This product is stable for at least 3 years from reception if used and stored under recommended conditions.

Quality Control

Bmax and Kd are determined using radioactive saturation binding assays (Figure 1). Protein concentration is determined using the BCA method ⁽¹⁾. Ratio-to-Reference (RTR) is determined by dividing the maximal signal of the current lot (Bmax in fmoles) by the maximal signal of a pre-defined reference tested in parallel. RTR is an indicator of lot-to-lot consistency. *We certify that these results meet our quality release criteria.

Ratio-to-Reference (RTR): 0.87
Expression Level (B_{MAX}): 17.021 pmol/mg membrane protein.
 K_D for [125 I]-(-)Iodocyanopindolol : 0.81 nM
Protein Concentration: 10 μ g/ μ L

(1) Smith, P.K., et al. (1985). Anal. Biochem. **150**, 76-85.

Recommended Assay Conditions

- Assay Buffer:** 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.02% ascorbic acid
- Wash Buffer:** 50 mM Tris-HCl pH 7.4
- Binding Protocol:** Binding assays are performed in 200 μ L total volume according to the following conditions:
- 1 - Membrane dilution: 0.05 mL of membranes + 7.45 mL assay buffer (1:150 dilution)
 - 2 - Incubation: 25 μ L of incubation buffer or (S)-(-)-Propranolol (Sigma P8688) 2 μ M final for non specific binding (Saturation binding assay)
For competition binding assay: 25 μ L of reference compounds at decreasing concentrations (see figure 2)
25 μ L of radioligand at the appropriate concentration (see graph below)
150 μ L of diluted membranes
 - 3 - Incubation time: 60 minutes at 27 $^{\circ}$ C
 - 4 - Filtration: aspirate and wash 9 x 500 μ L with ice cold wash buffer over GF/C filter (presoaked in 0.5 % PEI).

Lot Specific Data

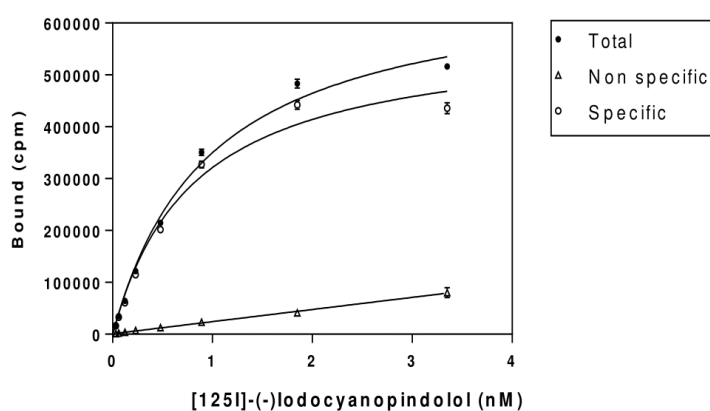


Figure 1: Saturation binding assay curve (filtration)
96-well saturation binding assay curve (10 μ g membranes/well, TopCount®) using [125 I]-(-)-Iodocyanopindolol (PerkinElmer NEX189 Lot No.: CW30740)

Typical Product Data

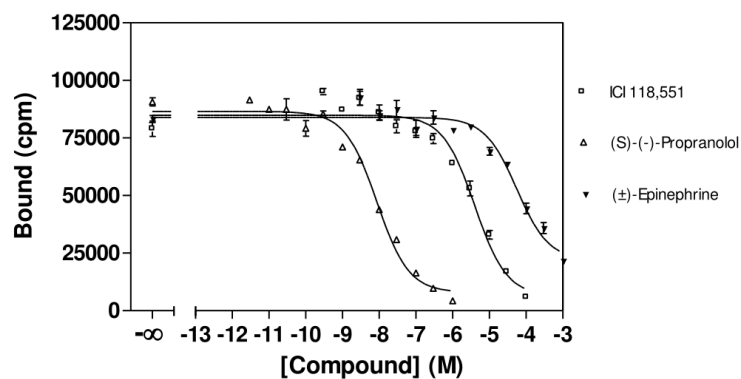


Figure 2: Competition binding assay curve (filtration)

96-well competition binding assay curve (10 μ g membranes/well, TopCount®). Recommended radioligand concentration = 0.25 nM.

*Even though two sites can be observed occasionally with some ligands, the data presented is derived from single site fitting.

Reference Compounds	K _i (nM)
ICI 118,551	3533
(S)-(-)-Propranolol	7.6
(±)-Epinephrine	48950

Suggested Materials and Instrumentation

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human Adrenergic β_2 Receptor

Product No.: RBHBE2M400UA

Lot No.: 495-130-A

Material Provided

Membranes: 1 x 400 units / 400 μ L frozen aliquot

Product Information

Cellular Background: HEK293-EBNA**GenBank Accession Number:** J02960**Unit Size:** 1 μ g protein / unit**Storage Buffer:** 50 mM Tris-HCL (pH 7.4), 0.5mM EDTA, 10mM MgCl₂, 10% sucrose.**Storage Conditions:** Store at -80°C. **Freeze-thaw is not recommended** as it can affect product performance and homogeneity. In order to minimize negative impact of freeze-thawing, flash freeze in liquid nitrogen for 30 seconds prior to transferring to -80°C.**Stability:** This product is stable for at least 3 years from reception if used and stored under recommended conditions.

Quality Control

B_{max} and K_d are determined using radioactive saturation binding assays (Figure 1). Protein concentration is determined using the BCA method ⁽¹⁾. Ratio-to-Reference (RTR) is determined by dividing the maximal signal of the current lot (B_{max} in fmoles) by the maximal signal of a pre-defined reference tested in parallel. RTR is an indicator of lot-to-lot consistency. *We certify that these results meet our quality release criteria.

Ratio-to-Reference (RTR): N/A**Expression Level (B_{MAX}):** 3.8 pmol/mg membrane protein.**K_D for [¹²⁵I]-(-)Iodocyanopindolol:** 0.075 nM**Protein Concentration:** 1 μ g/ μ L

(1) Smith, P.K., et al. (1985). *Anal. Biochem.* **150**, 76-85.

Recommended Assay Conditions

- Assay Buffer:** 50 mM Tris-HCl pH 7.4, 20 mM MgCl₂, 2mM EGTA
- Wash Buffer:** 50 mM Tris-HCl pH 7.4
- Binding Protocol:** Binding assays are performed in 200 μ L total volume according to the following conditions:
- 1 - Membrane dilution: 0.05 mL of membranes + 7.45 mL assay buffer (1:150 dilution)
 - 2 - Incubation: 25 μ L of incubation buffer or ICI 118,551 (Sigma I127) 10 μ M final for non specific binding (Saturation binding assay)

For competition binding assay: 25 μ L of reference compounds at decreasing concentrations (see figure 2)

25 μ L of radioligand at the appropriate concentration (see graph below)
150 μ L of diluted membranes
 - 3 - Incubation time: 60 minutes at 27 °C
 - 4 - Filtration: aspirate and wash 9 x 500 μ L with ice cold wash buffer over GF/C filter (presoaked in 0.5 % PEI).

Lot Specific Data

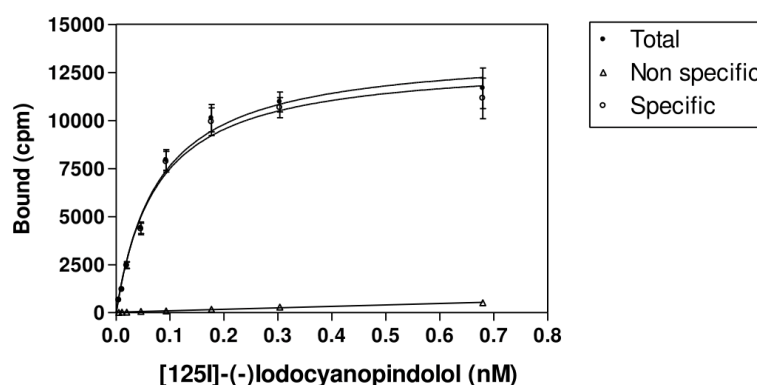


Figure 1: Saturation binding assay curve (filtration)
96-well saturation binding assay curve (1 μ g membranes/well, TopCount®) using [¹²⁵I]-(-)-iodocyanopindolol (PerkinElmer NEX189 Lot No.: CW41180)

Typical Product Data

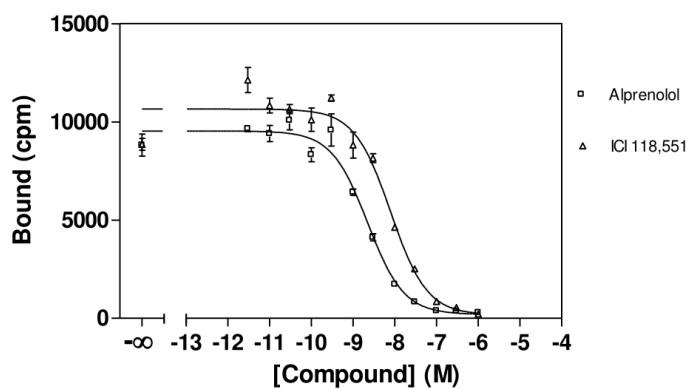


Figure 2: Competition binding assay curve (filtration)

96-well competition binding assay curve (1 μ g membranes/well, TopCount®). Recommended radioligand concentration = 0.2 nM.

*Even though two sites can be observed occasionally with some ligands, the data presented is derived from single site fitting.

Reference Compounds	K_i (nM)
Alprenolol	0.57
ICI 118,551	2.0

Suggested Materials and Instrumentation

Please visit our website

www.perkinelmer.com/GPCR

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**TECHNICAL
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Membrane Target Systems™

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human Adrenergic β_3 Receptor
Product No.: ES-035-M400UA
Lot No.: 1732440
Material Provided
Membranes: 1 x 400 units / 400 μ L frozen aliquot

Product Information
Cellular Background: CHO-K1

GenBank Accession Number: X70811

Unit Size: 2 μ g protein / unit

Storage Buffer: 50 mM Tris-HCL (pH 7.4), 0.5mM EDTA, 10mM MgCl₂, 10% sucrose.

Storage Conditions: Store at -80°C. **Freeze-thaw is not recommended** as it can affect product performance and homogeneity. In order to minimize negative impact of freeze-thawing, flash freeze in liquid nitrogen for 30 seconds prior to transferring to -80°C.

Stability: This product is stable for at least 3 years from reception if used and stored under recommended conditions.

Quality Control

Bmax and Kd are determined using radioactive saturation binding assays (Figure 1). Protein concentration is determined using the BCA method ⁽¹⁾. Ratio-to-Reference (RTR) is determined by dividing the maximal signal of the current lot (Bmax in fmoles) by the maximal signal of a pre-defined reference tested in parallel. RTR is an indicator of lot-to-lot consistency. *We certify that these results meet our quality release criteria.

Ratio-to-Reference (RTR): 0.77

Expression Level (B_{MAX}): 0.98 pmol/mg membrane protein

K_D for [¹²⁵I]-(-)Iodocyanopindolol : 0.71 nM

Protein Concentration: 2 μ g/ μ L

(1) Smith, P.K., et al. (1985). *Anal. Biochem.* **150**, 76-85.

Recommended Assay Conditions

- Assay Buffer:** 25 mM Hepes pH 7.4, 1 mM EDTA, 0.5% BSA
- Wash Buffer:** 10 mM Hepes pH 7.4, 500 mM NaCl
- Binding Protocol:** Binding assays are performed in 200 μ L total volume according to the following conditions:
- 1 - Membrane dilution: 0.05 mL of membranes + 7.45 mL assay buffer (1:150 dilution)
 - 2 - Incubation: 25 μ L of incubation buffer or Cyanopindolol (Tocris 0993) 250 μ M final for non specific binding (Saturation binding assay)

For competition binding assay: 25 μ L of reference compounds at decreasing concentrations (see figure 2)

25 μ L of radioligand at the appropriate concentration (see graph below)
150 μ L of diluted membranes
 - 3 - Incubation time: 30 minutes at 27 $^{\circ}$ C
 - 4 - Filtration: aspirate and wash 9 x 500 μ L with ice cold wash buffer over GF/B filter (presoaked in 0.5 % BSA).

Lot Specific Data

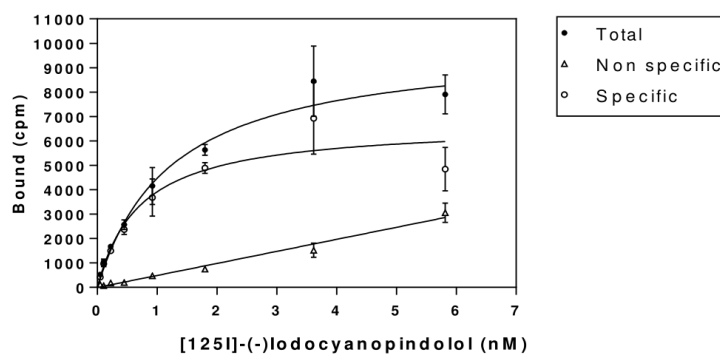


Figure 1: Saturation binding assay curve (filtration)
96-well saturation binding assay curve (2 μ g membranes/well, TopCount®) using [125 I]-(-)iodocyanopindolol (PerkinElmer NEX189 Lot No.: CW30830)

Typical Product Data

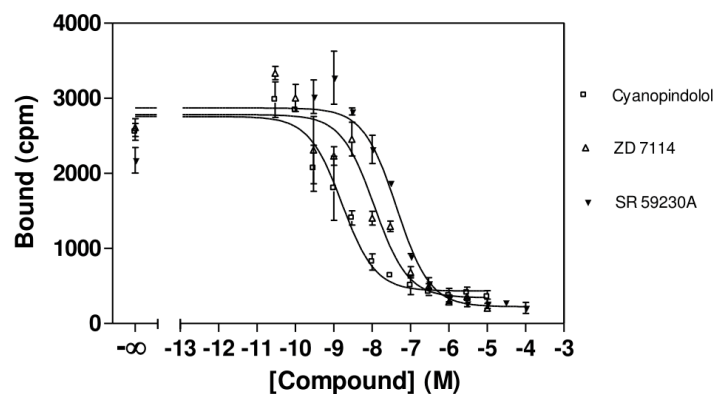


Figure 2: Competition binding assay curve (filtration)

96-well competition binding assay curve (2 μ g membranes/well, TopCount®). Recommended radioligand concentration = 0.165 nM.

*Even though two sites can be observed occasionally with some ligands, the data presented is derived from single site fitting.

Reference Compounds	K _i (nM)
Cyanopindolol	1.2
ZD 7114	8.6
SR 59230A	33

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