Synthesis and pharmacological characterization of dibenzodiazepinone-type heterodimeric and fluorescently labeled muscarinic receptor ligands

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Xueke She
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Prof. Dr. Armin Buschauer (1st Referee)
Prof. Dr. Günther Bernhardt (2nd Referee)
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Professional Training:

02/2014  Radioanalytical working methods for pharmacists. Regensburg, Germany.

12/2013-03/2017 Associated member of the Research Training Group (Graduiertenkolleg 1910) “Medicinal Chemistry of Selective GPCR Ligands” of the German Research Foundation. Regensburg, Germany.

06/2014-04/2017 Member of the Emil Fischer Graduate School of Pharmaceutical Sciences and Molecular Medicine. Regensburg, Erlangen, Germany.
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Chapter 1

General Introduction
1. General Introduction

1.1. G-protein coupled receptors

1.1.1. GPCRs as drug targets and their classification

The superfamily of G-protein coupled receptors (GPCRs) is one of the largest and most studied families of proteins, over 800 GPCRs are encoded in the human genome\textsuperscript{1-2}. A major characteristic of GPCR proteins is that they have seven α-helical transmembrane domains, an extracellular N-terminus and an intracellular C-terminus connected by three intracellular and extracellular loop domains (\textit{cf.} Figure 1). Diverse kinds of endogenous ligands bind to GPCRs, such as biogenic amines, peptides, amino acids, glycoproteins, prostanoids, phospholipids, fatty acids, nucleosides, nucleotides and Ca\textsuperscript{2+} ions, as well as pheromones, fragrances or flavors are recognized by sensory 7TM receptors\textsuperscript{3}. Moreover, the endogenous ligands of around 140 GPCRs are not identified, these GPCRs are the so-called orphan GPCRs\textsuperscript{4}, this field is relatively wide open for new discoveries. GPCR agonist and antagonist drugs have therapeutic benefit across a broad spectrum of human diseases\textsuperscript{5}, like peptic ulcers, pain, asthma, schizophrenia, depression and hypertension. According to sequence homology and functional roles, the mammalian members of this superfamily can be classified into three major families: A, B and C. Family A, also referred as the rhodopsin-like family, represents the largest and best studied subgroup of 7TM receptors. It includes aminergic and some peptidergic GPCRs as well as receptors addressed by nucleotides, lipids and other small molecules\textsuperscript{6}. The other two main subfamilies are the family B (secretin receptor family) and metabotropic glutamate receptors, γ-aminobutyric acid receptors and the Ca\textsuperscript{2+} sensing receptor for family C\textsuperscript{7}. The receptor topology varies between the families, e.g. with respect to the location of the orthosteric binding domain (OBD). For family A, the OBD is located within the 7TM domains, for family B in the large extracellular loop regions, and for family C the OBD is existent at the extracellular Venus-flytrap-like domain\textsuperscript{8}.

The crystal structure of bovine rhodopsin gave the first insight into the three-dimensional architecture of GPCRs\textsuperscript{9}. Further structures were solved, e.g. those of the human β\textsubscript{2}-adrenoceptor\textsuperscript{10-11}, the turkey β\textsubscript{1}-adrenergic receptor\textsuperscript{12}, the human adenosine 2A receptor\textsuperscript{13}, the dopamine D\textsubscript{3} receptor\textsuperscript{14}, opsin\textsuperscript{15-16} and the chemokine CXCR4 receptor\textsuperscript{17}. The crystal structures of GPCRs provided insights into the molecular mechanisms of GPCR activation and constitutive activity and served as template for GPCR homology models to study GPCR conformations and ligand receptor interactions. So far, the structures of more than 30 different GPCRs have been solved.
Figure 1. Schematic structure of a generic GPCR. GPCRs all contain a common core composed of seven transmembrane helices (7TM) with an extracellular N-terminal domain and an intracellular C-terminal domain. The TMs are connected by three extracellular loops (EL1-EL3) and three intracellular loops (IL1-IL3). (modified from the literature)

1.1.2. GPCR signaling pathways

To explain the interaction between a GPCR (R), its ligand (A) and the respective G-protein (G), several models have been proposed. The ternary complex model was firstly described by DeLean and colleagues. In this model, the binding of the activated receptor to membrane proteins such as G-proteins was taken into account, the process is:

\[
A + R \quad \xrightarrow{\alpha} \quad AR + G \quad \xrightarrow{\gamma} \quad ARG
\]

However, the ternary complex model was not able to explain the phenomena such as constitutive activity or inverse agonism. Refinement of the ternary complex model resulted the extended ternary complex model, which additionally implies the equilibrium between the inactive \(R_i\) and the active \(R_a\) receptor states. The active state receptor can form a complex with G-protein (G) to \(R_aG\), or agonist activation can induce a ternary complex \(AR_aG\). The term \(\alpha\) refers to the multiple differences in affinity of the ligand for \(R_a\) over \(R_i\), and \(\gamma\) refers to the multiple difference in affinity of the receptor for G-protein, when the ligand is bound to the receptor (cf. Figure 2A). Moreover, further refinements were made with the cubic ternary complex (CTC) model. The concept of the CTC is shown in Figure 2B. Accordingly, receptors are assumed to exist in inactive (\(R_i\)) and active (\(R_a\)) conformations, which may or may not be coupled to G-protein. At equilibrium, four receptor species namely \(R_i\), \(R_a\), \(R_iG\), and \(R_aG\) make up the native ensemble. \(K_G\), \(K_{act}\), and \(\beta\) are defined as the interconversions between these four species (\(R_i\), \(R_a\), \(R_iG\), and \(R_aG\)). Each of the four receptor species in the native ensemble can bind to ligand (refers to A). The equilibrium dissociation constants for the binding of ligand to the members of the native ensemble are \(K_{A_i}\), \(\alpha K_{A_i}\), \(\gamma K_{A_a}\), and \(\delta \alpha \gamma K_{A_a}\), respectively.
Figure 2. (A) Illustration of the extended ternary complex model\textsuperscript{19} discrimination between $R_i$ and $R_a$. (B) Scheme of the cubic ternary complex model of GPCR ligand-receptor interactions. The cubic ternary complex model comprises eight distinct types of receptor species: $R_i$, $R_a$, $AR_i$, $AR_a$, $R_a G$, $R_i G$, $AR_i G$, and $AR_a G$ ($R_i$ = inactive receptor, $R_a$ = activated receptor, $A$ = ligand, $G$ = G-protein). (modified from literature\textsuperscript{21-23})

Upon activation (agonist-dependent or independent), GPCRs can transduce signals into cells through G-Protein coupling. There are two main classes of G-proteins, small cytoplasmic G-proteins and heterotrimeric G-proteins\textsuperscript{24-26}. In the latter case, $\alpha$, $\beta$ and $\gamma$ subunits constitute a heterotrimeric G-protein. Agonist binding to extracellular or transmembrane domains of a GPCR leads to the stabilization of a certain receptor conformation resulting in binding of the intracellular receptor domains to a heterotrimeric G protein. This agonist-receptor-G-protein complex, is termed ternary complex. Upon receptor activation, the GDP-bound G-protein interacts with the intracellular face and C-terminus of the receptor, inducing GDP to GTP exchange on the $G\alpha$ subunit and concurrent dissociation of the activated $\alpha$ subunit ($G\alpha^*$) from the $\beta\gamma$-dimer\textsuperscript{6}. Both G-protein subunits regulate the activity of enzymatic effectors, such as adenylate cyclases, phospholipase C isoforms, and ion channels, to regulate the production and release of small molecule ‘second messengers’. The receptor returns to the inactive state by intrinsic GTPase activity of the $G\alpha$ subunit: cleavage of the terminal $\gamma$-phosphate of GTP, resulting in GDP, gives the inactive GDP-bound $G\alpha$ and the subunits re-associate allowing a new cycle\textsuperscript{24, 27-28}. A scheme of the G-protein cycle is depicted in Figure 3.
Figure 3. The G-protein cycle. RGS, regulator of G-protein signaling. (adopted and modified from the literature\textsuperscript{29})

More than twenty G-protein α-subunits have been described for mammalian systems. Based on the degree of primary sequence similarities and different regulation of effectors\textsuperscript{30}, G-protein α-subunits can be divided into 4 families, termed as G\textsubscript{as}, G\textsubscript{ai/o}, G\textsubscript{aq} and G\textsubscript{12/13}\textsuperscript{3, 24, 29}. Stimulation of the G\textsubscript{as} subfamily activates AC1-9, leading to an increase in intracellular cAMP (3’-5’-cyclic adenosine monophosphate) levels, and consequently, to an activation of protein kinase A (PKA) or the mitogen-activated protein kinase (MAPK) pathway, which results in a modulation of gene transcription\textsuperscript{31}. Activation of the G\textsubscript{ai} family results in an inhibition of AC5 and AC6, and thus in decreased intracellular cAMP formation. Proteins of the G\textsubscript{aq} family activate phospholipases C\textsubscript{β1-3} (PLC\textsubscript{β}), leading to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) into 1,2-diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP\textsubscript{3}). IP\textsubscript{3} mediates the release of Ca\textsuperscript{2+} from intracellular compartments in particular from the endoplasmic reticulum. DAG activates protein kinase C (PKC) which phosphorylates of various proteins\textsuperscript{32}. It has been difficult to selectively study the cellular processes mediated by G\textsubscript{12} and G\textsubscript{13}, for G\textsubscript{12}/G\textsubscript{13} as the respective receptors are often simultaneously stimulated by activating members of the G\textsubscript{q/11}-family. It was reported that G\textsubscript{12}/G\textsubscript{13} stimulates a Na\textsuperscript{+}/H\textsuperscript{+} exchange and alters a variety of downstream effectors including phospholipase A\textsubscript{2} (PLA\textsubscript{2})\textsuperscript{33-34}. Like the GTP-bound α-subunits, the β- and γ-subunits, forming a tightly associated βγ-complex, are also able to interact with effector proteins such as PLC\textsubscript{β} and ion channels\textsuperscript{24} and regulate their functions. Besides the modulation of GPCR signaling by ligand (agonist) binding, the signaling is also substantially influenced by receptor expression, desensitization, and internalization in response to binding of different ligands\textsuperscript{35}. The desensitization of GPCRs occurs through molecular mechanisms, involving phosphorylation of activated receptors by G protein-coupled
receptor kinases (GRKs). The phosphorylation leads to binding of β-arrestin preventing receptor-G-protein interactions while allowing activation of arrestin-dependent signaling pathways. β-Arrestin can also induce receptor internalization, this regulates the level of cell surface receptors, thus effecting signaling to downstream effector pathways.

1.1.3. Allosteric modulation of GPCRs

The word *allosteric* comes from the Greek *allos*, and the arrangement of atoms in space refers *steric*. The term ‘orthosteric ligand’ describes a receptor ligand, which binds to the binding site of the endogenous ligand, activating the receptor as agonist/partial agonist, blocking the actions of the endogenous agonist as an antagonist, or suppressing the receptor’s basal activity as an inverse agonist. Most GPCR drug discovery efforts to date have focused on targeting such sites. But as the orthosteric site is often highly conserved across subtypes of a given GPCR subfamily, this approach cannot always lead to highly subtype selective ligands. For example, the development of orthosteric selective ligands for one of the five subtypes of muscarinic acetylcholine receptors (M₁-M₅) is highly challenging. A number of MR agonists such as xanomeline, milameline, sabcomeline, cevimeline and talsaclidine were developed for the treatment of Alzheimer’s disease. However, due to their poor subtype selectivity and associated side effects, their use in clinical trials was limited. Meanwhile, in addition to orthosteric sites, potentially all GPCRs possess additional binding sites, which are designated allosteric sites. Ligands, which bind to an allosteric site of a GPCR, can potentially modulate the binding and/or signaling properties of the orthosteric ligand. Because they do not face the same evolutionary pressure as orthosteric sites, allosteric sites are less conserved than orthosteric sites, presenting novel avenues for achieving selectivity in drug action. Furthermore, allosteric modulators with limited positive or negative cooperativity will have a ceiling level to their effect, it means they might be potentially safer than orthosteric ligands if administered in high doses. Finally, given that allosteric modulators can promote a conformational change of the receptor, their ability to effect orthosteric ligand efficacy is not surprising. Some synthetic small molecules acting in such a pathway-selective allosteric modulation are actually reported.

Modulators binding to an allosteric site to stabilize a certain receptor conformation lead to an increase or decrease of the affinity and/or efficacy of an orthosteric agonist. Such kind of allosteric ligands are termed as positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs), respectively. Besides PAMs and NAMs, some allosteric ligands can be neutral (or silent), showing no cooperativity with the orthosteric ligand despite binding to an allosteric site of the receptor. With the discovery of allosteric modulators and the intricate
mechanisms underlying their pharmacological properties, classical receptor models need to be revised or expanded. The allosteric ternary complex model (ATCM), which also forms the basis for many quantitative studies of GPCR allostery, is considered as the simplest model to describe allosteric interactions. This model, describing the interactions between an orthosteric agonist, an allosteric modulator and a receptor, was derived from the original TCM, which describes ligand, receptor, and G-protein interactions. A prerequisite for an application of the ATCM is a simultaneous binding of the orthosteric ligand (A) and the allosteric ligand (B) to distinct, i.e. non-overlapping sites of the receptor. The ATCM provides estimates of the respective equilibrium dissociation constants, $K_A$ and $K_B$, as well as the “cooperativity factor” ($\alpha$), which is a measure of the mutual effect of the two ligands on each other’s affinity to the receptor. Values of $\alpha > 1$ indicate positive cooperativity. In this case an allosteric ligand promotes the binding of the orthosteric ligand. Values of $\alpha < 1$ refers to negative cooperativity, i.e. the allosteric modulator inhibits the binding of the orthosteric ligand, whereas $\alpha = 1$ means that binding of the allosteric ligand to the receptor does not alter the affinity of the orthosteric ligand. Provided that the two sites are conformationally linked, the orthosteric ligand will modulate the binding of the allosteric ligand in the same way.

Figure 4C and 4D illustrate another important aspect of such allosteric interactions, namely the fact that these effects are limited, in other words, they are saturable. The extent of the effect is defined by the numerical value of $\alpha$ (the lower the deviation of $\alpha$ from unity, the less pronounced is the modulatory effect of B).

**Figure 4.** (A) The allosteric ternary complex model (ATCM), which describes the interaction of two ligands (e.g., an orthosteric agonist and an allosteric modulator) on a receptor in terms of their equilibrium dissociation constants ($K_A$, $K_B$) and the cooperativity ($\alpha$) between the ligands. (B) Effect of a competitive antagonist on orthosteric ligand receptor occupancy ($pA$), simple competitive interactions are characterized by mutually exclusive binding of the two ligands for the same site and, thus, allow for a theoretically limitless dextral shift of orthosteric ligand occupancy. (C) An allosteric enhancer ($\alpha = 10$) or (D) allosteric inhibitor ($\alpha = 0.1$) on orthosteric ligand receptor occupancy ($pA$) based on the simple
ternary complex model for allosteric interactions. In these examples, ligand affinity is either maximally diminished or enhanced by a factor of 10. (adopted and modified from the literature\textsuperscript{41-42})

1.2. Muscarinic acetylcholine receptors

1.2.1. Muscarinic receptors subtypes and signaling pathways

Muscarinic acetylcholine receptors (mAChRs or MRs) belong to class A (rhodopsin-like) GPCRs. In humans, the family of mAChRs comprises five subtypes (M\textsubscript{1}-M\textsubscript{5}). The M\textsubscript{1}, M\textsubscript{3} and M\textsubscript{5} subtypes couple to the \(G_{q/11}\) family of G proteins, resulting in phospholipase C activation, hydrolysis of PIP\textsubscript{2} and an increase in intracellular Ca\textsuperscript{2+} (cf. Figure 5A). The M\textsubscript{2} and M\textsubscript{4} subtypes couple to the \(G_{i/o}\) family, resulting in the inhibition of adenylyl cyclase with a decrease in cAMP formation\textsuperscript{45,51} (cf. Figure 5B).

![Diagram of signaling pathways of muscarinic receptors](image)

**Figure 5.** Schematic illustration of the signaling pathway of muscarinic receptors. (adopted and modified from the literature\textsuperscript{29})

MRs are widely distributed in the periphery and the central nervous system (CNS), and play important roles in many functions of the CNS. The M\textsubscript{1} subtype is widely expressed in forebrain regions, including the cerebral cortex, hippocampus and striatum\textsuperscript{52-55}. It was reported that reduced M\textsubscript{1}R signaling in the CNS is associated with cognitive deficits such as Alzheimer’s disease\textsuperscript{56-57}. Selective agonists of the M\textsubscript{1} mAChR have been pursued as a potential avenue for the treatment of dementia-related disorders\textsuperscript{58}. The M\textsubscript{2} mAChRs is located, e.g. in the brainstem, hypothalamus/thalamus, hippocampus, striatum, cortex\textsuperscript{52-53} and in the heart\textsuperscript{51}. It is suggested that elevated acetylcholine levels through antagonism of presynaptic M\textsubscript{2} muscarinic
autoreceptors may be beneficial in the treatment of psychosis and Alzheimer’s disease\textsuperscript{59-62}. In this respect, M\textsubscript{1}/M\textsubscript{2} selectivity is crucial as antagonism at post-synaptic M\textsubscript{1} receptors is counterproductive, as confirmed by studies with the non-selective antagonists such as scopolamine, which lead to cognitive deficits\textsuperscript{63}. In the heart, M\textsubscript{2} receptors may be directly linked through G proteins to ion channels devoid of a second messenger\textsuperscript{64-66}. M\textsubscript{3} mAChRs are expressed at low levels in the cortex, the striatum, the hippocampus and the hypothalamus/thalamus\textsuperscript{53, 67-68}. M\textsubscript{3} receptors are involved in regulating longitudinal growth by promoting the proliferation of pituitary somatotroph cells, suggesting the M\textsubscript{3}R as a target to treat growth disorders\textsuperscript{69}. Furthermore, M\textsubscript{3} receptors participate in the regulation of smooth muscle motility. The M\textsubscript{3} receptor antagonist solifenacin could be bladder-selective to provide new approaches to the pharmacotherapy of an overactive bladder\textsuperscript{70}. M\textsubscript{4} mAChRs are found in hippocampus, cortex, hypothalamus/thalamus and striatum\textsuperscript{65}. Selective M\textsubscript{4} antagonists could be used as a medication for parkinsonism by controlling the tremor associated with Parkinson’s disease\textsuperscript{71}. The M\textsubscript{5} mAChR, which is expressed at consistently low levels in the brain, is the least studied subtype among the five muscarinic receptors\textsuperscript{72}. The M\textsubscript{5} and the D\textsubscript{2} receptor were found to be co-localized within the pars compacta of the substantia nigra\textsuperscript{73}, and activation of the M\textsubscript{5}R was reported to facilitate the release of dopamine.

Physiologically, each muscarinic subtype is activated by the endogenous ligand acetylcholine (cf. Figure 6). Several naturally occurring ligands binding to muscarinic acetylcholine receptors were described, e.g. the agonist muscarine (where the receptors name comes from), and the antagonist atropine (cf. Figure 6).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{structures.png}
\caption{Structures of acetylcholine (endogenous MR agonist) and the naturally occurring ligands muscarine (MR agonist from the mushroom \textit{Amanita muscaria} L.) and atropine (MR antagonist from \textit{Atropa belladonna} L.).}
\end{figure}

For many years, pharmaceutical chemists aimed to find ligands, which bind selectively to one subtype of the MR family. As the five subtypes exhibit a high conservation of the orthosteric binding site, there are only very few subtype selective orthosteric agonists and antagonists. Many ligands exhibit merely a (weak) preference for one subtype. For example, the antagonist pirenzepine\textsuperscript{74} shows a preference for the M\textsubscript{1}R, the antagonists methoctramine, AF DX 384, tripitramine and himbacine show preference for the M\textsubscript{2}R\textsuperscript{75-77}, the antagonist darifenacin prefers
the M₂R⁷⁸, and the antagonist PD102807 prefers the M₄R⁷⁹. These ligands are used as pharmacological tools rather than for therapeutic purposes (cf. Figure 7).

**Figure 7.** Chemical structures of muscarinic receptor ligands reported to exhibit preference for one muscarinic receptor subtype.

### 1.2.2. Muscarinic receptor agonists

According to a general trend, MR agonists are small molecules, whereas antagonists are large molecules, often containing an aromatic moiety⁵¹. Arecoline (cf. Figure 8), which is a naturally-occurring MR agonist shows no subtype selectivity. Due to the ester group, being prone to hydrolysis, its clinical application was limited. To improve the pharmacological properties, for example, the ester group was replaced with more stable five-membered heterocyclic rings such as oxadiazole, thiazazole or tetrazole. The 1,2,5-thiadiazole derivative xanomeline (cf. Figure 8), which was reported as a cognition enhancer, potentially useful for the treatment of schizophrenia, has higher potency and efficacy for M₁ and M₄ than for M₂, M₃ and M₅ receptor subtypes⁸⁰. Although xanomeline prefers M₁R with positive effects on verbal learning and short-term memory function⁸¹, it causes side effects including nausea salivation and diaphoresis, presumably due to the activation of other mAChR subtypes⁸². Moreover, it was reported that xanomeline showed a unique mode of mAChR activation, being different from
Chapter 1

that of conventional agonists such as carbachol, and it was reported to bind to the M₁R in a wash-resistant manner, which may be attributed not only to hydrophobic interactions between xanomeline’s O-hexyl chain and the receptor, but also to the binding to a secondary binding site of the receptor. Replacement of the side-chain and the azacyclic ring of xanomeline led to the discovery of potent MR agonists. The phenylpropargylthio-1azabicyclo[3.2.1] octane endo analogue (cf. Figure 8) is the most selective M₁/M₂ compound in this series, which was suggested to be a drug candidate for AD. Many MR ligands, such as tritiated 3-quinuclidinyl benzilate ([³H]QNB), a non-selective MR antagonist, which is routinely used for receptor binding studies, contain a quinuclidine ring. The same holds for some moderately potent MR agonists, for example, talsaclidine is a quinuclidinyl-propargyl ether (cf. Figure 8), which is a selective M₁R agonist and has been reported for therapy of Alzheimer’s disease. Another “quinuclidine-related” M₁R agonist is SB202026, which contains a cyanooximether group in the structure (cf. Figure 8). As a functionally selective M₁R partial agonist, SB202026 was used in the investigation of the cholinergic hypothesis of senile dementia of the Alzheimer type (SDAT). To increase receptor subtype selectivity, several pharmacophores with increased rigidity were developed, for example, the conformationally restricted spiro compound RS-86 (cf. Figure 8), which was a M₂R preferable agonist, and some attempts also have been made to change the profile towards M₁R selectivity based on this structure. Oxotremorine (cf. Figure 8) has long been known as a partial M₁R agonist. By means of classical isosteric replacement of a methylene group with oxygen and exchange the pyrrolidine moiety of oxotremorine by a trimethylammonium group yielded a trimethylammonium salt related to oxotremorine (cf. Figure 8), which displayed binding affinity comparable to that of oxotremorine, but showed a pronounced selectivity for M₂R versus M₁R.

Figure 8. Chemical structures of selected muscarinic receptor agonists.

1.2.3. Allosteric modulation of muscarinic receptors
The lack of highly selective mAChR orthosteric ligands demands alternative approaches to develop subtype selective MR ligands. All five muscarinic receptor subtypes possess at least one extracellular allosteric binding sites for small molecules\textsuperscript{93-94}. The M\textsubscript{2} subtype was the first GPCR found to be sensitive to allosteric modulation\textsuperscript{95-96}, so that the M\textsubscript{2}R is one of the most extensively characterized allosteric model systems\textsuperscript{45}. The best studied allosteric modulators of the mAChRs are represented by neuromuscular-blocking agents, for example, gallamine, alcuronium, W84 and its heptamethylene congener C7/3-phth, and most of these prototypical common-site modulators have higher affinity to the M\textsubscript{2}R than to the other subtypes (cf. Figure 10). The allosteric behavior of gallamine can be demonstrated by equilibrium binding assays: Figure 9A and 9B show the effect of the modulator gallamine on the saturation binding of \[^{3}H\]NMS at the M\textsubscript{2}R expressed by CHO cells. Although gallamine is able to shift \[^{3}H\]NMS binding curves to the right, the allosteric nature of the interaction is revealed as progressively higher concentrations of gallamine fail to cause significant dextral displacements of the \[^{3}H\]NMS saturation curves, becoming obvious from curvilinear Schild regressions\textsuperscript{41}. Also the inhibitory effect of gallamine on equilibrium binding of \[^{3}H\]NMS could unmask the limited ability of this negative allosteric modulator to inhibit specific \[^{3}H\]NMS binding. From Figure 9C, it can be seen that when a low concentration of \[^{3}H\]NMS (0.1 nM) was applied, the increasing concentrations of gallamine resulted in an apparently complete inhibition of specific \[^{3}H\]NMS binding. However, applying a higher concentration of \[^{3}H\]NMS (2.0 nM), caused an incomplete \[^{3}H\]NMS binding by gallamine\textsuperscript{41}. Alcuronium was found allosterically increases \[^{3}H\]NMS binding at M\textsubscript{2} and M\textsubscript{4} subtypes; in contrast, it inhibits \[^{3}H\]NMS binding at M\textsubscript{1}, M\textsubscript{3} and M\textsubscript{5} subtypes\textsuperscript{97}. The alkaloid structure of alcuronium lead to the identification of related compounds such as strychnine, vincamine, eburnamonine, and brucine and its analogs as allosteric mAChR modulators\textsuperscript{98-99}.

**Figure 9.** (A) \[^{3}H\]NMS saturation binding curves obtained in the presence of the following concentrations of gallamine: 0 (●), 1 \(\mu\)M (○), 3 \(\mu\)M (□), 10 \(\mu\)M (◇) and 100 \(\mu\)M (△). (B) Effect of gallamine on the ratio of \[^{3}H\]NMS \(K_{d}\) values (“affinity-shift”) determined in the presence or absence of the modulator. (C) Inhibition of \[^{3}H\]NMS binding 0.1 nM (○) and 2.0 nM (●) by increasing concentrations of gallamine. Data were taken from the literature\textsuperscript{41}. 
In addition to the well-studied common mAChR allosteric site, Lazareno, Birdsall and colleagues defined a second allosteric site. For example, depending on the mAChR subtype, several indolocarbazole derivatives of staurosporine (cf. Figure 11) were found to show positive, negative and neutral cooperativity with Ach, but did not appear to interact with the prototypical modulators gallamine and brucine. Similarly, WIN 51,708 (cf. Figure 11) interacts with staurosporine in a competitive manner, whilst interacting with gallamine and strychnine in a noncompetitive way.

Given that allosteric modulators can induce a unique conformational change of the receptor, it is not surprising that they have the ability to affect orthosteric ligand efficacy. McN-A-343 (cf. Figure 12) was the mAChR agonist known to display functional selectivity, it was actually found to interact allosterically with [³H]NMS on M₂ mAChRs. Other agents were recently identified as potential mAChR allosteric agonists, such as AC-42 and its close structural
analog 77-LH-28-1. Both AC-42 and 77-LH-28-1 (cf. Figure 12) display high selectivity to activate the M₁ mAChR over other mAChR subtypes. TBPB (cf. Figure 12) was reported as a novel highly selective agonist for the M₁ receptor with no agonist activity at any other mAChR subtypes, mutagenesis and molecular pharmacology studies revealed that TBPB activates M₁R through an allosteric site rather than the orthosteric ACh binding site. A highly selective and efficacious allosteric potentiator for M₁R is BQCA (benzyl quinolone carboxylic acid) (cf. Figure 12). N-desmethyloclozapine (cf. Figure 12), the major metabolite of the antipsychotic clozapine, which is a functionally-selective M₁ mAChR agonist that has been suggested to act allosterically.

![Chemical structures](image)

**Figure 12.** Examples of allosteric mAChR agonists (McN-A-343, AC 42, N-desmethyloclozapine, TBPB and 77-LH-28-1) and allosteric potentiators (BQCA).

### 1.2.4. Crystal structures of muscarinic receptors

Kobilka et al. reported the crystal structures of the M₂R (with bound antagonist QNB) and M₃R (with bound inverse agonist tiotropium) in 2012. The overall structure of the M₂R is similar to that of the M₃R, structural conservation includes intracellular loops (ICLs) 1 and 2, and extracellular loops (ECLs) 1-3, which share highly similar despite low sequence conservation. The binding poses of QNB (M₂R) and tiotropium (M₃R) were very similar, suggesting this pose to be a conserved binding mode: the ligands are deeply buried within the TM receptor core and covered by a ‘lid’ consisting of three conserved tyrosines, which separate the orthosteric site from the extracellular vestibule. The structures of the inactive M₂ and M₃ receptor suggested that these receptors possess a large extracellular vestibule, which was shown to be addressed by allosteric modulators. In this region of the receptor, the structural diversity is much higher than in the region of the orthosteric binding site. When an allosteric ligand binds to this site, an influence on the association and disassociation rates of orthosteric ligands are
expected. Recently, the $M_1$ and the $M_4$ receptor were crystallized in the inactive state bound with the inverse agonist tiotropium bound. The structures of the $M_1$ and $M_4$ receptors are similar to the previously solved inactive states of $M_2$ and $M_3$ receptors, with similar positioning of the seven-transmembrane (TM1-7) bundle and root mean squared deviations of 0.6-0.9 Å\textsuperscript{107-108}. By comparing structures of the $M_1$ and the $M_4$ receptor, subtle differences between the receptors are observed on the extracellular and intracellular sides corresponding to regions that are least conserved across the MR subtypes\textsuperscript{109}. Further comparison of the orthosteric sites of $M_1R$ and $M_4R$ revealed the $M_4R$ is closer related to the $M_1$ than to the $M_2$ subtype. Another breakthrough was the solution of the active-state structure of the $M_2R$ in the presence of the high affinity agonist iperoxo and the positive allosteric modulator LY2119620\textsuperscript{110}. This structure revealed that LY2119620 binds to the vestibule just above the orthosteric agonist iperoxo. Moreover, it suggested that the allosteric modulator induces structural changes such as the inward motion of TM6, which directly contacts the allosteric modulator, the orthosteric agonist, and probably, the G protein as well\textsuperscript{110}. The MR crystal structures offer important insights into the activation mechanism and allosteric modulation of MRs, and are supposed to contribute to the development of highly selective MR ligands.

![Figure 13. Muscarinic M_3 receptor (PDB code 4U14\textsuperscript{108}) bound with tiotropium at the orthosteric pocket. The orthosteric and allosteric sites are indicated in yellow and blue elliptical shaded areas, respectively. Image was generated by UCSF Chimera\textsuperscript{111}. (adopted and modified from the literature\textsuperscript{108})](image)

### 1.3. Bivalent ligands

Much of the pioneering work describing bivalent ligands for GPCRs was led by the group of Portoghese\textsuperscript{112-115}, targeting opioid receptor subtypes. Bivalent ligands are defined as compounds containing two pharmacophores, which are covalently tethered by a linker. Bivalent ligands are divided into two general classes: homobivalent ligands, containing two
identical pharmacophores, and heterobivalent ligands, wherein the two pharmacophores are different\textsuperscript{113, 116-117}. A number of homo- and heterobivalent GPCR ligands have been developed in recent years, such as histamine receptor\textsuperscript{118-119}, dopamine receptor\textsuperscript{120-123}, muscarinic receptor\textsuperscript{124-125}, adenosine receptor\textsuperscript{126-127} and serotonin receptor\textsuperscript{128-129} ligands. There are several hypotheses to explain the enhanced activity and selectivity observed for some bivalent GPCR ligands. The first possibility is through a univalently bound state, the unbound recognition unit being in the locus of neighboring binding sites, in another word, the bivalent ligand increased local concentration of free pharmacophore, which increases the probability of a productive binding event (\textit{cf}. Figure 14A). Secondly, the bivalent ligand interacts simultaneously with two orthosteric binding sites of a dimeric or oligomeric complex of GPCRs, leading to increased affinity and selectivity (\textit{cf}. Figure 14B), and thirdly, the bivalent ligand occupies the orthosteric binding site and a second (low affinity) allosteric binding site at one and the same receptor protomer (\textit{cf}. Figure 14C).

![Figure 14](image)

**Figure 14.** Possible binding modes of bivalent ligands. (A) Increased local concentration of free pharmacophore. (B) Induction of GPCR dimerization. (C) Simultaneous binding to an orthosteric and allosteric binding site of the same receptor molecule (bitopic or dualsteric binding mode). (adopted and modified from the literature\textsuperscript{130})

A new concept in drug design derived from bivalent ligand was focusing on bitopic or dualsteric ligands, these ligands can address both the orthosteric and the allosteric site of a receptor protein\textsuperscript{131-132}. The reasons to pursue a bitopic ligand approach are various. The most important reason is that a bitopic ligand can theoretically achieve improvements in affinity and selectivity. Another advantage is that the use of pure allosteric modulators relies on the presence of endogenous agonist tone to mediate their effects, whereas the use of a bitopic ligands would engage the orthosteric site irrespective of the presence or absence of endogenous tone\textsuperscript{133}. Examples of rationally designed bitopic MR ligands are oxotremorine-related hybrid molecules\textsuperscript{134}. These hybrid ligands target the mAChRs, constructed with the agonist oxotremorine (an orthosteric pharmacophore) and hexamethonium derived allosteric modulators (the structure of one of these hybrid ligands will be shown in Chapter 3). Though these hybrid ligands didn’t show improved affinity, they gained subtype selectivity as compared with the parent orthosteric agonist (oxoremorine). Mohr and colleagues linked a non-selective, orthosteric agonist iperoxo, with a M\textsubscript{2}-selective bis(ammonio)alkane-type allosteric fragment to form hybrid 2 (\textit{cf}. Figure 15). This approach engendered receptor subtype selective
activation compared with the parent agonist. A bitopic ligand was developed for the adenosine A<sub>1</sub> receptor (A<sub>1</sub>R) by linking a positive allosteric modulator and an orthosteric pharmacophore through a spacer of 9 carbon atoms. The resulting bivalent ligand LUF6258 (cf. Figure 15) did not show significant changes in affinity or potency in the presence of an allosteric enhancer (PD81,723), taken a hint that the bivalent ligand bridges both sites on the receptor. Recently, Christopoulos and colleagues linked the endogenous orthosteric agonist adenosine with a PAM of the A<sub>1</sub> adenosine receptor VCP171, leaded the rational design of a bitopic A<sub>1</sub> adenosine receptor ligand VCP746, which displays biased agonism relative to prototypical A<sub>1</sub>AR ligands (cf. Figure 15). More examples of bitopic ligands such as THRX-160209 will be shown in Chapter 3.

Figure 15. Examples of bitopic GPCR ligands. The non-selective, orthosteric, agonist iperoxo and a M<sub>2</sub> selective bis(ammonio)alkane-type allosteric fragment were linked to form hybrid 2; Bitopic ligands LUF6258 and VCP746 target the adenosine A<sub>1</sub> receptor.

1.4. Radioligands for muscarinic receptor

Several radioligands were developed as pharmacological tools for studying the receptor binding of MR ligands. For instance, the orthosteric antagonists [³H]QNB, [³H]N-methyl scopolamine, [³H]pirenzepine and [³H]tiotropium, the orthosteric agonists [³H]acetylcholine and [³H]oxotremorine-M have been used as radioligands. Moreover, [³H]NMS has been frequently used to identify allosteric interactions of agents, and to check the common allosteric site hypothesis for the allosteric modulators alcuronium, W84, and gallamine. One important approach was the development of [³H]dimethyl-W84, the first radiolabeled allosteric modulator of the M<sub>2</sub> mAChR. Table 1 shows the pK<sub>d</sub> values of the muscarinic receptor radioligands mentioned above.
Table 1. Reported pKd values of radioligands binding to muscarinic mAChRs.

<table>
<thead>
<tr>
<th>Radioligands</th>
<th>pKd M1R</th>
<th>pKd M2R</th>
<th>pKd M3R</th>
<th>pKd M4R</th>
<th>pKd M5R</th>
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<td>10.1 - 10.6</td>
<td>10.4</td>
<td>9.7 - 10.5</td>
<td>10.2 - 10.7</td>
</tr>
<tr>
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<td>9.3 - 9.9</td>
<td>9.7 - 10.2</td>
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<td>-</td>
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<td>-</td>
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<tr>
<td>[3H]tiotropium</td>
<td>-</td>
<td>10.3</td>
<td>10.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>8.7</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>[3H]acetylcholine</td>
<td>-</td>
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<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>[3H]dimethyl-W84</td>
<td>8.5</td>
<td></td>
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</tbody>
</table>

* pKd values reported in the literature (data taken from the IUPHAR/BPS database (guidetopharmacology.org) in Nov. 2016).

1.5. Fluorescently labeled GPCR ligands

The major disadvantages of radiolabeled ligands are that they are potentially hazardous to human health, high costs of production and waste disposal and the requirement of special laboratory conditions. Besides label-free ligand binding assays such as affinity selection mass spectrometry (AS-MS)\textsuperscript{148} and nuclear magnetic resonance spectroscopy (NMR)-based binding assays\textsuperscript{149}, fluorescent ligand-based receptor binding assays were developed for several peptide and non-peptide GPCRs as an alternative to radioligand binding assays. Like radioligands, fluorescent ligands can be used to determine the affinity of unlabeled receptor ligands\textsuperscript{150}. Moreover, fluorescent ligands have been used for the screening of small molecules (fragments) in terms of GPCR binding. For instance, one fluorescent ligand was used in a fluorescence resonance energy transfer (FRET)-based assay to identify the first small molecule agonist for the apelin receptor, which may lead to new molecules being developed for the treatment of heart failure\textsuperscript{151}. New non-peptide small molecule ligands for the formylpeptide receptor were identified from a library of 880 compounds by using a fluorescein labelled formylmethionine-leucine-phenylalanine-lysine based on high-throughput flow cytometry (HTFC)\textsuperscript{152}. In addition, fluorescent ligands can be used to study receptor expression patterns, GPCR localization and internalization as demonstrated for the μ and δ opioid receptor\textsuperscript{153} and AT\textsubscript{1} angiotensin receptor\textsuperscript{154}, as well as ligand-receptor binding kinetics in living cells as demonstrated for the adenosine-A\textsubscript{1} and -A\textsubscript{3} receptor\textsuperscript{155-156}. Fluorescent ligands can also be used to investigate the dynamics of GPCR oligomerization in living cells\textsuperscript{157-158}.

Several fluorescent ligands were also developed as useful pharmacological tools for studying muscarinic receptors. For example, Cu(I)-catalyzed 1,3-dipolar cycloaddition, referred to as
“click” chemistry, was used to incorporate of a fluorophore (Lissamine Rhodamine B) into a MR antagonist scaffold derived from pirenzepine. The high affinity and very slow off-rates of the telenzepine analogs Cy3B-telenzepine (Kd = 35 pM, M1R) and Alexa488-telenzepine (Kd = 0.5 nM, M1R) were applied to investigate the dynamics of M1 receptor dimerization in living cells. BODIPY-pirenzepine was used to study pirenzepine-induced formation of muscarinic M1R dimers. A fluorescent tolterodine-BODIPY (boron dipyrromethene) conjugate was described to exhibit affinity for M1, M2 and M4 receptors in the nanomolar range, harboring the potential to be used for high throughput screening. However, a slow degradation of this compound was observed even when stored in the dark (t1/2 = 10 weeks). Ilien and co-workers prepared pirenzepine derivatives linked to the boron-dipyrromethene [Bodipy (558/568)] fluorophore via spacers of varying lengths and coupled the lissamine rhodamine B fluorophore (in para position) to AC42 to study bitopic fluorescent ligands.

1.6. References


74. Wess, J.; Lambrecht, G.; Mutschler, E.; Brann, M.; Dörje, F., Selectivity profile of the


Chapter 1


Chapter 2

Scope and objectives
Muscarinic acetylcholine receptors (MRs) were identified as targets for the treatment of disorders such as Alzheimer’s and Parkinson’s disease. The family of MRs comprises five subtypes and due to the high conservation of the orthosteric binding pocket, highly subtype selective ligands are lacking. As the MRs’ ‘vestibule’ or ‘common’ allosteric site is less conserved, the dualsteric ligand approach was considered a promising strategy to develop ligands with high subtype selectivity. The design of subtype selective MR ligands is supposed to benefit from the structural information provided by the recently reported crystal structures of the M₁R-M₅R₁ - ₄.

The recent study on a series of dibenzodiazepinone-type MR ligands revealed that the replacement of the terminal basic diethylamino moiety of the M₂R preferring antagonist DIBA (cf. Figure 1A) by various bulky groups afforded derivatives with high M₂R affinity. Based on these results, this doctoral thesis aimed at the synthesis and characterization of DIBA-derived heterodimeric MR ligands by conjugation of the DIBA pharmacophore to different reported orthosteric and allosteric MR ligands (the agonist xanomeline, the antagonists 4-DAMP and propantheline, and the allosteric modulators TBPB and 77-LH-28-1) using different linker types (cf. Figure 1B). In addition, monomeric and homodimeric ligands had to be prepared as reference compounds.

The heterodimeric ligands had to be investigated with respect to M₁R-M₅R affinity using the orthosteric radioligand [³H]NMS to obtain the MR selectivity profiles. Furthermore, the preparation and characterization of radio- and fluorescence labeled compounds had been envisaged. For this purpose, amino-functionalized branched linker elements (cf. Figure 1B) had to be incorporated into the dimeric ligands.

M₂R saturation binding studies (including experiments in the presence of allosteric MR modulators) as well as competition binding experiments with various kinds of MR ligands (orthosteric, dualsteric, allosteric) had to be performed aiming at an elucidation of the binding mode of the heterodimeric ligands at the M₂R.
Figure 1. (A) Structure of DIBA and proposed site of attachment (blue arrow) for the conjugation to various MR ligands. (B) Moieties to be implemented in the linkers of dimeric MR ligands.

The characterization of selected fluorescently labeled ligands had to be substantiated by flow cytometry, high content analysis and fluorescence microscopy. From such investigations it was expected to get information on the binding mode at the M2R and the suitability of the fluorescent ligands with respect to the characterization of unlabeled MR ligands as well as cellular imaging.

2.1. References


Chapter 3

Synthesis and pharmacological characterization of dibenzodiazepinone-type heterodimeric muscarinic receptor ligands
3. Synthesis and pharmacological characterization of dibenzodiazepinone-type heterodimeric muscarinic receptor ligands

3.1. Introduction

GPCRs constitute a large superfamily of cell surface receptors, which are classified into more than 100 subfamilies. GPCRs are the most frequently addressed drug targets. Around 30% of all marketed prescription drugs act on GPCRs. Muscarinic acetylcholine receptors (mACHRs or MRs) belong to the class A GPCRs and comprise five receptor subtypes in humans, designated M₁R to M₅R. M₁R, M₃R, M₅R receptors couple to G proteins of the Gq class, M₂R, M₄R receptors interact with G proteins of the Gi family. MRs have been identified as targets for the treatment of various diseases such as Alzheimer’s disease and Parkinson’s disease, but due to the high conservation within the orthosteric binding site across all five mAChR subtypes, the development of subtype selective orthosteric ligands is challenging.

Besides the ACh binding pocket (the “orthosteric site”), muscarinic receptors contain additional “allosteric binding sites” which are less conserved than the orthosteric region and can potentially be exploited to develop subtype selective ligands. The M₂ subtype was the first GPCR to be found as being sensitive to allosteric modulation and numerous allosteric MR modulators were identified, such as compounds 14, 15 and 16 (cf. Figure 1B).

Homo- and heterobivalent ligands have been emerged at GPCRs field in recent years, like histamine, dopamine, and adenosine and NPY receptors. Benefits of bivalent ligands can include increased ligand affinity and improved selectivity. As a variation of bivalent ligands, the concept of dualsteric (bitopic orthosteric/allosteric) ligands of GPCRs led to novel chemical tools. The first examples of rationally designed bitopic ligands targeting mAChRs were derived from the orthosteric agonist oxotremorine (3) and hexamethonium-like allosteric modulators. This series of hybrid molecules showed a gain in subtype selectivity compared to 3 (e.g. compound 17, cf. Figure 1C). Another example of a designed bitopic MR ligand is THRX-160209 (compound 18, cf. Figure 1C), which is reported to exhibit higher affinity and subtype selectivity than the corresponding monovalent ligands and was suggested to bind in a multivalent manner to the M₂ receptor.

AF-DX 384³¹ (8) (cf. Figure 1A) was developed from the M₁ preferring M receptor antagonist pirenzepine as M₂R preferring pyridobenzodiazepinone-type M receptor antagonist. Likewise, the dibenzodiazepinone-type M receptor antagonist DIBA (9) showed high affinity for the M₂ receptor (Kᵢ value: 0.3 nM). Moreover, Tränkle et. al suggested a dualsteric binding
mode of 8 at the M₂ receptor\(^{34}\). A hybrid MR ligand formed of 8 and the allosteric modulator W84 (15) was reported to exhibit a pronounced positive cooperativity with NMS, pointing a new way for the development of allosteric enhancers\(^{35-36}\).

Figure 1. (A) Chemical structures of reported MR agonists (1-3) and antagonists (4-11). (B) Chemical structures of the allosteric MR ligands 12-16. (C) Structures of the hybrid ligands 17 and 18, which were suggested to bind dual sterically at the M₂R\(^{29-30}\).

This study was directed towards the design, synthesis, and pharmacological evaluation of DIBA-derived heterodimeric MR ligands comprising five combinations of DIBA with reported orthosteric or allosteric MR ligands: ‘DIBA-xanomeline’, ‘DIBA-TBPB’, ‘DIBA-77-LH-28-1’, ‘DIBA-4-DAMP’ and ‘DIBA-propantheline’ (in total nineteen heterodimeric ligands). Xanomeline (cf. Figure 1A) is a M₁ selective MR agonist (see general introduction). TBPB (cf. Figure 1B) was reported to selectively activate M₁ receptors through an allosteric mechanism as shown by mutagenesis and molecular pharmacology studies\(^{37-39}\). And TBPB was proposed
as a bitopic ligand, interacting with both the orthosteric site and an allosteric site at the M₁R⁴⁰.
Similarly, the competitive mechanism or with a strong negative cooperativity between [³H]NMS and 77-LH-28-1 (cf. Figure 1B) was reported at M₁R, and 77-LH-28-1 was also suggested to bind allosterically to [³H]NMS-occupied M₁ receptor, and competed with the prototypical allosteric modulator (C₇₃-phth)⁴¹. 4-DAMP and propantheline (cf. Figure 1A) are non-selective orthosteric MR antagonists with high affinities (Kᵢ (4-DAMP, M₁R-M₅R): 0.52-3.80 nM, Kᵢ (propantheline, M₁R-M₅R): 0.057-0.33 nM)³¹, ⁴².

In addition to the heterodimeric ligands, four homodimeric ligands derived from xanomeline, two homodimeric ligand derived from DIBA, a monomeric DIBA derivative, a monomeric xanomeline derivative and a monomeric TBPP derivative were prepared as reference compounds. Furthermore, two radiolabeled heterodimeric ligands ('DIBA-TBPB' type and 'DIBA-xanomeline' type) were prepared as molecular tools and characterized by saturation binding (including experiments in the presence of allosteric modulators “Schild-like analysis”), kinetic investigations and equilibrium competition binding studies.

3.2. Results and discussion
3.2.1. Chemistry
3.2.1.1. Synthesis of the diazepane derivative 28

Treatment of diethyl oxalate (19) with one equivalent of benzylamine (20) afforded the N-substituted ethyl oxamate 21. The subsequent reaction with methylamine converted compound 21 to the unsymmetrical N,N'-disubstituted oxamide 22. Reduction of 22, using lithium aluminum hydride, resulted in compound 23 as reported⁴³. Homopiperazine 25 was obtained from 23 by nitro-Mannich reaction using nitroethanol and paraformaldehyde. Compound 25 was treated with an excess of tert-C₄H₉OK in methanol resulted compound 26⁴⁴-⁴⁶. Reduction of the nitro group in 26 to an amino group using Raney nickel, and subsequent Boc-protection gave compound 27. Debenzylation of 27 applying palladium-catalyzed hydrogenolysis yielded compound 28 (cf. Scheme 1).

Scheme 1. Synthesis of the diazepane derivative 28. Reagents and conditions: (a) CHCl₃, reflux, overnight, 65%; (b) CH₃NH₂ (2M in THF), EtOH, rt, 8 h, 97%; (c) LiAlH₄, abs. THF, 0 °C/reflux, overnight,
60%; (d) paraformaldehyde, toluene/EtOH 1:1 (v/v), reflux, 6 h, 88%; (e) potassium tert-butoxide, MeOH, 40 °C, 30 min, 67%; (f) H₂, Ni-Raney, EtOH, rt, overnight, 98%; (g) di-tert-butyl dicarbonate, CHCl₃, rt, overnight, 54%; (h) 10% Pd/C, H₂, THF/H₂O 1:4 (V/V), rt, overnight, 77%.

3.2.1.2. Synthesis of the piperazine derivative 32

The synthesis of piperazine derivative 32 started with commercially available 2-bromoethan-1-amine hydrobromide (29), which was Boc-protected to obtain compound 30. This intermediate was treated with an excess of piperazine (31) to afford building block 32 (cf. Scheme 2).

Scheme 2. Synthesis of the piperazine derivative 32. Reagents and conditions: (a) di-tert-butyl dicarbonate, triethylamine, CH₂Cl₂, rt, overnight, 80%; (b) K₂CO₃, MeCN, reflux, 3 h, 91%.

3.2.1.3. Synthesis of xanomeline derivatives 40, 41, 46 and 52-55

Hydroxy- or amino-functionalized xanomeline derivatives (38 and 50) were required as key intermediates for the synthesis of heterodimeric ligands (cf. Scheme 3). The intermediate 34 was synthesized from 3-pyridinecarbaldehyde (33) according a slightly modified Strecker synthesis from the literature. Initially, 33 was treated with potassium cyanide yielding the cyanohydrin, after isolation, the cyanohydrin was immediately reacted with ammonium chloride under basic aqueous conditions to give 34. The intermediate 34 was cyclized with sulfur monochloride in DMF to give 3-(3-chloro-1,2,5-thiadiazol-4-yl)pyridine (35). Propane-1,3-diol (36) or pentane-1,5-diol (42) were treated with sodium hydride and 35 to give the hydroxylated derivatives 37 and 43. This procedure is different from the described nucleophilic displacement of the chlorine of 35, where one of the hydroxyl groups was protected with tert-butylchlorodiphenylsilane (TBDPSCl). 37 and 43 were quaternized with excess methyl iodide in acetone and then reduced to the tetrahydropyridine product 38 or 44 with the help of sodium borohydride in methanol (cf. Scheme 3). In order to convert the primary hydroxy groups of 38 and 44 to bromide, an Apple reaction was applied using tetrabromomethane and triphenylphosphine in dichloromethane to yield compounds 39 and 45. Alkylation of piperazine using the bromides 39 or 45 gave the intermediate 40 and the homodimeric ligand 46. When compound 28 alkylated with compound 39, followed by removal of the Boc group, afforded the monomeric ligand 41, which was prepared for comparative purposes in pharmacological assays.

The synthesis of amino-functionalized derivatives of xanomeline is shown in Scheme 3,
deprotonation of tert-butyl (5-hydroxypentyl)carbamate (47) with excess of sodium hydride in anhydrous tetrahydrofuran followed by refluxing in the presence of intermediate 35 afforded compound 48. Reduction and methylation of 48 led to the 1,2,5,6-tetrahydropyridine derivative 49. Cleavage of the Boc group using trifluoroacetic acid (TFA) in dichloromethane afforded compound 50, which was coupled with compound 51 by using coupling reagents TBTU and HOBt in the presence of DIPEA to obtain intermediate 52 after subsequent removal of the Boc group. Furthermore, compound 50 was treated with octanediol dichloride or decanediol dichloride in the presence of triethylamine to yield the homodimeric compounds 53 or 54. Amidation of terephthalic acid with amine 50 using coupling reagents EDC and HOBt afforded the homodimeric ligand 55, containing a rigid central linker moiety (cf. Scheme 3).

**Scheme 3.** Synthesis of the xanomeline derivatives 39, 40, 41, 46 and 52-55. Reagents and conditions: (a) KCN, H₂O, AcOH, 5 °C/rt, 2 h, 98%; (b) NH₄Cl, 25% aq NH₃, rt, 20 h, 67%; (c) S₂Cl₂, DMF, 5-10 °C, 45 min, 69%; (d) NaH, abs. THF, reflux, 2-8 h, 52% for 37, 48% for 43, 27% for 48; (e) (1) methyl iodide, acetone, rt, 24-36 h; (2) NaBH₄, MeOH, 0 °C/rt, overnight, 41% for 38, 92% for 44, 79% for 49; (f) CBr₄, PPh₃, CH₂Cl₂, -5 °C/rt, 24 h, 50% for 39, 82% for 45; (g) K₂CO₃, MeCN, microwave, 110 °C (30 min) or reflux (2 h), 66% for 40, 22% for 46; (h) (1) 28, K₂CO₃, MeCN, microwave, 110 °C (30 min); (2) TFA/CH₂Cl₂ 1:4 v/v, rt, 8 h, 66%; (i) TFA/CH₂Cl₂ 1:4 v/v, rt, 8 h, 56%; (j) (1) 51, TBTU, HOBt, DIPEA,
DMF, rt/60 °C, 3 h; (2) TFA/CH$_2$Cl$_2$ 1:4 v/v, rt, 8 h, 89%; (k) octanediol dichloride or decanediol dichloride, triethylamine, abs. THF, 0 °C/rt, overnight, 39% for 53, 65% for 54; (l) terephthalic acid, EDC, HOBr, DMF, rt, overnight, 26%.

3.2.1.4. Synthesis of TBPB derivatives 63, 64, 66, 67

The synthesis of TBPB derivatives is outlined in Scheme 4. Beginning with commercially available 1-fluoro-2-nitrobenzene (56), an aromatic nucleophilic substitution with ethyl 4-aminopiperidine-1-carboxylate (57) under microwave irradiation yielded nitroaniline 58, which was reduced by palladium-catalyzed hydrogenation to provide o-phenylenediamine derivative 59. The benzimidazolone formation from 59 using triphosgene afforded 60 (cf. Scheme 4). Subsequent removal of the ethyl carbamate group by alkaline hydrolysis gave 61.$^{51}$ Reductive amination of the N-Boc protected piperidinone (62) with the secondary amine 61, using sodium cyanoborohydride, followed by removal of the Boc group afforded compound 63. Alkylation of 63 with tert-butyl (2-bromoethyl)carbamate (30) and Boc-deprotection yielded 64 as a building block for the synthesis of heterodimeric ligands. Acylation of 63 with 2-bromoacetyl bromide (65) afforded amide 66 (cf. Scheme 4). Finally, N-alkylation of the homopiperazine derivative 28 with bromide 66 followed by removal of Boc group afforded the TBPB derivative 67.

Scheme 4. Synthesis of the TBPB derivatives 63, 64, 66, 67. Reagents and conditions: (a) K$_2$CO$_3$, NaI, DMF, microwave, 180 °C, 10 min, 72%; (b) 10% Pd/C, H$_2$, rt, overnight, 89%; (c) triphosgene, NaHCO$_3$, CH$_2$Cl$_2$, 0 °C/rt, 2 h, 73%; (d) 10% aq NaOH, reflux, 5 h, 81%; (e) (1) 62, NaBH$_3$CN, acetic acid, MeOH, 0 °C/rt, overnight; (2) TFA/CH$_2$Cl$_2$ 1:4 v/v, rt, 8 h, 75%; (f) (1) 30, K$_2$CO$_3$, MeCN, reflux, 8 h; (2) TFA/CH$_2$Cl$_2$ 1:4 v/v, rt, 8 h, 86%; (g) 2-bromoacetyl bromide, pyridine, CHCl$_3$, 0 °C/rt, overnight, 91%; (h) (1) 28, K$_2$CO$_3$, MeCN, microwave, 110 °C, 30 min; (2) TFA/CH$_2$Cl$_2$ 1:4 v/v, rt, 8 h, 43%. 
3.2.1.5. Synthesis of 4-DAMP derivatives 71 and 73

The synthesis of the building blocks 71 and 73, required for the preparation of 'DIBA-4-DAMP'-type heterodimeric ligands, started from diphenylacetic acid (68) and Boc protected piperidin-4-ol (69) (cf. Scheme 5). Unlike a reported procedure, which transformed the carboxylic acid 68 into the corresponding acid chloride prior to esterification with alcohol 69, the ester 70 was formed from 68 and 69 using N,N'-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) as coupling reagents. Treatment of 70 with TFA gave 71 as the bisidesmethyl analogue of 4-DAMP. Alkylation of 71 with bromide 30 afforded compound 72, which was converted to 73 by removal of the Boc group (cf. Scheme 5).

Scheme 5. Synthesis of the 4-DAMP derivatives 71 and 73. Reagents and conditions: (a) DCC, DMAP, CH₂Cl₂, 0 °C/rt, overnight, 97%; (b) TFA/CH₂Cl₂ 1:4 v/v, rt, 8 h, 56% for 42, 83% for 44; (c) K₂CO₃, MeCN, reflux, 3 h, 67%.

3.2.1.6. Synthesis of propantheline derivatives 77, 78 and 80

Scheme 6 depicts the synthesis of the propantheline building blocks 77, 78 and 80, which were used for the synthesis of the 'DIBA-propantheline'-type heterodimeric ligands. Xanthene-9-carboxylic acid (74) was condensed with 2-bromoethan-1-ol or 4-bromobutan-1-ol to yield compound 75 and 76, respectively. Treatment of 75 and 76 with an excess of piperazine afforded the alkylation products 77 and 78 in moderate yield (cf. Scheme 6). Alkylation of 77 with bromide 30 gave compound 79, which was converted to the propantheline-derived compound 80 by Boc-deprotection using TFA.
Scheme 6. Synthesis of the propantheline derivatives 77, 78 and 80. Reagents and conditions: (a) 2-bromoethanol or 4-bromobutan-1-ol, DCC, DMAP, CH₂Cl₂, 0 °C/rt, overnight, 68% for 75, 56% for 76; (b) K₂CO₃, MeCN, reflux, 1.5 h or overnight, 59% for 77, 46% for 78; (c) K₂CO₃, MeCN, reflux, 2 h, 57%; (d) TFA/CH₂Cl₂ 1:4 v/v, rt, overnight, 88%.

3.2.1.7. Synthesis of 77-LH-28-1 derivatives 86 and 88

The synthesis of the 77-LH-28-1-derived intermediates 86 and 88 is shown in Scheme 7. Treatment of commercially available 3,4-dihydro-2(1H)-quinolinone (81) with 1-chloro-3-iodopropane (82) in the presence of caesium carbonate in acetonitrile afforded compound 83 as described in the literature. Alkylation of piperidine 84 with chloride 83 in the presence of potassium carbonate and sodium iodide yielded compound 85. The alcohol 85 was converted to the corresponding bromide (86) under Apple reaction condition using tetrabromomethane and triphenylphosphine (cf. Scheme 7). Compound 86 was treated with piperazine 32 to afford the alkylation product 87, which was Boc-deprotected to yield the 77-LH-28-1 derivative 88.

Scheme 7. Synthesis of the 77-LH-28-1 derivatives 86 and 88. Reagents and conditions: (a) Cs₂CO₃, MeCN, 50 °C, 12 h, 69%; (b) K₂CO₃, NaI, MeCN, 50 °C, 24 h, 53%; (c) CBr₄, PPh₃, CH₂Cl₂, -5 °C/rt, overnight, 31%; (d) K₂CO₃, MeCN, reflux, 2 h, 62%; (e) TFA/CH₂Cl₂ 1:4 v/v, rt, 8 h, 97%.
3.2.1.8. Synthesis of DIBA derivatives 94, 95 and 96

The preparation of the dibenzodiazepinone derivatives 94, 95, and 96 is illustrated in Scheme 8. The dibenzodiazepinone moiety 91 was obtained by heating 2-chlorobenzoic acid (89) and o-phenylenediamine (90) in chlorobenzene in the presence of copper\(^{54-55}\). Compound 91 was treated with chloroacetyl chloride to give the acylated derivative 92 (cf. Scheme 8). Nucleophilic substitution of the chlorine in 92 by 4-(piperidin-4-yl)butan-1-ol (84) gave compound 93. The alcohol 93 was converted to the respective bromide (compound 94) by using the Apple reaction conditions described above. Alkylation of compound 28 using bromide 94 and subsequent Boc-deprotection yielded the monomeric DIBA derivative 95 (cf. Scheme 8). Aiming at dimeric ligands with extended linkers, compound 32 was alkylated using again bromide 94, followed by Boc-deprotection with TFA to obtain compound 96.

![Scheme 8. Synthesis of the DIBA derivatives 94, 95 and 96. Reagents and conditions: (a) copper powder, chlorobenzene, reflux, 6 h, 13%; (b) 2-chloroacetyl chloride, \(N,N\)-dimethylaniline, THF, overnight, 84%; (c) \(K_2CO_3\), MeCN, reflux, 8 h, 62%; (d) CBr\(_4\), PPh\(_3\), CH\(_2\)Cl\(_2\), -5\(^\circ\)C/rt, overnight, 78%; (e) (1) 28, \(K_2CO_3\), MeCN, reflux, 3 h; (2) TFA/CH\(_2\)Cl\(_2\)/H\(_2\)O 10:10:1 v/v/v, rt, 2 h, 17%; (f) (1) 32, \(K_2CO_3\), MeCN, reflux, 3 h; (2) TFA/CH\(_2\)Cl\(_2\) 1:4 v/v, rt, 8 h, 66%.](image)

3.2.1.9. Synthesis of dibenzodiazepinone-derived heterodimeric ligands 97-102, 105a, 106-108, 110, 111 and 114-120

The synthesis of the dibenzodiazepinone-derived heterodimeric ligands is outlined in Scheme 9 (97-101, 105a, 106-108) and Scheme 10 (110, 111, 114-120). The heterodimeric ligands 97-101 were prepared through alkylation of compounds 40, 63, 71, 77 and 78, respectively, using bromide 94. The heterodimeric ligand 102 was prepared by alkylation of compound 10\(^{55}\) (cf. Figure 1A) with bromide 86 (cf. Scheme 9). Alkylation of the homopiperazine derivative 103 applying a mixture of the bromides 39 and 94, followed by Boc-deprotection, yielded the heterodimeric ligand 105a, which was propionylated to give the congener 106. Likewise,
alkylation of 103 applying a mixture of the bromides 66 and 94, followed by Boc-deprotection, gave the heterodimeric ligand 107, which was propionylated yielding the congener 108. The DIBA type homodimeric ligand 105b was isolated during the preparation of compound 105a and 107, and the non-DIBA type homodimeric ligands formed by double alkylation of 103 with bromides 39 and 66 were not isolated (cf. Scheme 9).

Scheme 9. Synthesis of the DIBA-derived heterodimeric ligands 97-102, 105-108. Reagents and conditions: (a) K$_2$CO$_3$, MeCN, microwave, 110 °C, 30 min or reflux, 3 h to overnight, 41% for 97, 57% for 98, 27% for 99, 51% for 100, 38% for 101; (b) NaI, K$_2$CO$_3$, MeCN, reflux, 3 h, 52% for 102; (c) TFA/CH$_2$Cl$_2$/H$_2$O 10:10:1 v/v/v, rt, 2 h, 17% for 105a (two steps), 12% for 107 (two steps); (d) DIPEA, DMF, rt, 2 h, 95% for 106, 96% for 108.
Scheme 10. Synthesis of the dibenzodiazepinone-type homo- or heterodimeric ligands 110-120. Reagents and conditions: (a) (1) TBTU, HOBt, diisopropylethylamine, DMF, 3 h; (2) TFA/CH$_2$Cl$_2$/H$_2$O 10:10:1 v/v/v, rt, 2 h, 14% for 110, 10% for 114, 28% for 116, 15% for 118, 4% for 120; (b) DIPEA, DMF, rt, 2 h, 89% for 111, 79% for 113, 88% for 115, 83% for 117, for 86% 119.

Amidation of the isophthalic acid derivative 109 applying a mixture of amines 96 and 52, followed by Boc-deprotection, afforded the heterodimeric ligand 110 and the homodimeric ligand 112. Propionylation of 110 and 112 gave the congeners 111 and 113, respectively (cf. Scheme 10). Likewise, the heterodimeric ligands 114, 116, 118 and 120 were obtained by amidation of 109 using the amine mixtures 96/64, 96/73, 96/80 and 96/88, respectively, and subsequent Boc-deprotection (cf. Scheme 10). Propionylation of 114, 116 and 118 at the
central linker moiety afforded the propionamide congeners 115, 117 and 119. It should be noted that the respective non-DIBA type homodimeric ligands generated by double amidation of 109 with amines 52, 64, 73, 80 and 88 were formed, but were not isolated (cf. Scheme 10).

3.2.2. Equilibrium competition binding studies with \[^{3}\text{H}]\text{NMS}\)

The receptor binding affinities of twenty eight new MR ligands, comprising the monomeric xanomeline derivative 41, TBPB derivative 67, DIBA derivative 95, the ‘DIBA-DIBA’ type homodimeric ligands 112 and 113, the ‘xanomeline-xanomeline’ type homodimeric ligands 46 and 53-55, as well as the ‘DIBA-xanomeline’ type heterodimeric ligands 97, 105a, 106, 110 and 111, the ‘DIBA-TBPB’ type heterodimeric ligands 98, 107, 108, 114 and 115, the ‘DIBA-77-LH-28-1’ type heterodimeric compounds 102 and 120, the ‘DIBA-propantheline’ type dimeric ligands 100, 101, 118 and 119, and the ‘DIBA-4-DAMP’ type heterodimeric ligands 99, 116 and 117, were investigated in equilibrium binding experiments using the orthosteric antagonist radioligand \[^{3}\text{H}]\text{N}-\text{methylscopolamine (}[^{3}\text{H}]6\) at live CHO cells stably expressing the human muscarinic receptor subtypes M1-M5. M1-M5 receptor saturation binding experiments with \[^{3}\text{H}]6\) were performed in our lab55. The pKd values amounted to 9.85 (M1R), 10.1 (M2R), 10.1 (M3R), 10.5 (M4R) and 9.63 (M5R) were in good agreement with previously reported data31, 56.

Figure 2A shows the sigmoidal curves of a subset of the xanomeline-type homodimeric ligands 46 and 53-55, the monomeric ligand 41 derived from xanomeline, the monomeric ligand 95 derived from DIBA, and five ‘DIBA-xanomeline’ type heterodimeric ligands (97, 105a, 106, 110 and 111) at M2R. For the homobivalent ligands of xanomeline, we observed a gradual, spacer-length-dependent (not counting the xanomeline O atom) increase in affinity at the M2R for the 14- (46), 18- (55), 20- (53), and 22-atom (54) spacers (K values: 21, 4.6, 4.0 nM and 2.3 nM, respectively) compared to the parent compound xanomeline (K = 210 nM) (data not shown in Table 1), the covalent tethering of two xanomeline pharmacophores caused 10-100 fold higher affinities at M2R compared with xanomeline (2). A similar phenomenon was observed for M1R, compared with the parent compound xanomeline (K = 160 nM, data not showed in Table 1), ligands 46, 55, 53 and 54 showed increasing M1R affinities (K values: 53, 24, 5.9 and 4.5 nM, respectively) depending on the length of the linker, and showed 3-35 fold higher affinities at M1R than xanomeline (2). In contrast, the monomeric ligand derived from xanomeline (compound 41) obtained from replacing xanomeline’s O-hexyl chain by the amino-functionalized moiety showed a decrease in affinity at M2R.

All of the tested DIBA derivatives (including monomeric (95) and heterodimeric (97, 105a, 106,
110 and 111) ligands) exhibited $K_i$ values in the low nanomolar range at M2R, and showed higher affinities at M2R than xanomeline derived monomeric (41) or homodimeric (46, 53-55) ligands (cf. Table 1). Compound 97, containing a xanomeline and a DIBA moiety linked by piperazine, showed a remarkably high M2R affinity with a $K_i$ value of 0.08 nM. Compound 105a, containing 1,4-diazepane ring between DIBA and xanomeline moieties, showed low nanomolar range affinity with a $K_i$ value of 0.51 nM. With a $K_i$ value of 0.46 nM, the propionamide congener 106 had a comparable affinity. Remarkably, though compound 110 and its propionamide derivative 111 comprise complex linker moieties, these two heterodimeric ligands had high affinity at the M2R with $K_i$ values of 0.26 nM and 0.35 nM, respectively. This means that, although the linkers in compounds 97, 105a, 106, 110 and 111 differ in the chemical nature and vary in length, these compounds showed comparable high M2R affinities (cf. Table 1). Moreover, all ‘DIBA-xanomeline’ type heterodimeric ligands showed slightly higher M2R affinities than the DIBA-derived monomeric ligand 95 ($K_i$ 2.2 nM), indicating that conjugation to the second pharmacophore (xanomeline) is favorable with respect to M2 receptor binding.

The effect on [3H]NMS M2R equilibrium binding of the second subset of compounds, comprising the monomeric ligand 67 derived from TBPB, the ‘DIBA-TBPB’ type ligands 98, 107, 108, 114 and 115, as well as the ‘DIBA-77-LH-28-1’ type heterodimeric compounds 102 and 120 is depicted in Figure 2B. The heterodimeric ligands (98, 102, 107, 108, 114, 115 and 120) all exhibited slightly higher M2 affinities ($K_i$ values: 0.20 to 0.82 nM) than the DIBA derived monomeric ligand 95 ($K_i$ value: 2.2 nM). The TBPB-like monomeric ligand 67 displayed low M2R affinity ($K_i$ 1800 nM). This phenomenon suggested that chemical dimerization of DIBA and TBPB, as well as DIBA and 77-LH-28-1 leads to increased affinity at M2R. Interestingly, the heterodimeric ligands containing a piperazine moiety in the spacer (114, 115, 120) showed slightly increased affinities with $K_i$ values in the range of 0.20-0.37 nM, compared to other heterodimeric ligands (98, 102, 107 and 108) without a piperazine moiety in the spacer ($K_i$ values from 0.60 to 0.82 nM), indicating that the involvement of piperazine moiety in the linker favors the interaction with the M2 receptor in this set of compounds.

The effect on [3H]NMS M2R equilibrium binding of the third subset of compounds, comprising the ‘DIBA-4-DAMP’ type heterodimeric ligands 99, 116 and 117, the ‘DIBA-propantheline’ type heterodimeric ligands 100, 101, 118 and 119 as well as the ‘DIBA-DIBA’ homodimeric ligands 112 and 113 are depicted in Figure 2C. The homo- or heterodimeric ligands, which contain long and basic spacers (118, $K_i = 0.80$ nM; 119, $K_i = 1.1$ nM; 116, $K_i = 0.84$ nM; 117, $K_i = 1.2$ nM), had higher M2 affinity compared to compounds with a short spacer (99, $K_i = 2.2$ nM; 100, $K_i = 2.1$ nM; 101, $K_i = 5.8$ nM). Moreover, the DIBA-derived homodimeric ligands 112 and 113,
showed comparable affinities at M2R ($K_i$ value: 0.15 nM or 0.58 nM) with the heterodimeric ligands 116-119, which also contained complex spacer moieties. This suggested that the ligands with bulky spacer moieties are even better tolerated by the M2R than ligands containing the sample linkers in the sets of ‘DIBA-4-DAMP’ or ‘DIBA-propantheline’ type heterodimeric ligands.

A selection of the new dibenzodiazepinone heterodimeric ligands (compounds 97, 98, 99, 100, 101, 102, 106, 108, 111, 115, 117, 119, 120) as well as the dibenzodiazepinone-type monomeric ligand 95 were also studied in equilibrium binding experiments with [3H]NMS at the M receptor subtypes M1, M3, M4 and M5. The $K_i$ values and hill slopes are included in Table 1. The [3H]NMS displacement curves of ligand 97, 106 and 115 at intact CHO-hMx cells (x = 1-5) are showed in Figure 2D-2F. All of these compounds showed a preference for the M2 receptor, but high M2R selectivity toward all of the other four subtypes wasn’t found for any of the MR ligands. Affinities obtained for the subtypes M1 and M4 were higher than affinities for the subtypes M3 and M5. There was one exception, compound 100, which showed the affinity pattern M2>M1=M5>M3>M4. For the rest of compounds, the selectivity pattern can be concluded as M2>M1>M4>M3=M5 (98, 99, 101, 102, 106, 111, 117); M2>M1=M4>M3>M5 (97, 115, 119, 120) and M2>M1>M4>M5>M3 (95, 108). With the excellent $K_i$ value of 0.08 nM at the M2R, compound 97 showed the best M2R selectivity among the investigated ligands, the $K_i$ value of 97 at the M1R was 23-fold higher, at the M4R 31-fold higher and the affinity to M3R and M5R was considerably lower with $K_i$ ratios of 175 and 425, respectively. Compared to 97, the MR antagonist tripitramine57, containing three pyridobenzodiazepinone moieties, and the monomeric pyridobenzodiazepinone derivative AF-DX 384 (compound 8, cf. Figure 1A)31 exhibit lower M2R selectivities according to published data ($K_i$ ratios M1R/M2R/M3R/M4R/M5R: 6:1:142:24:125 and 5:1:11:1.5:90, respectively).
Figure 2. (A-C) Concentration-dependent effects of compounds 41, 46, 53-55, 67, 95, 97-102, 105a, 106-108, 110-120 on [³H]NMS (c = 0.2 nM) equilibrium binding at intact CHO-hM₂ cells. (D-F) Concentration-dependent effects of the heterodimeric ligands 97 (D), 106 (E) and 115 (F) on equilibrium binding of [³H]NMS (c = 0.2 nM (M₁, M₂, M₃), 0.1 nM (M₄) or 0.3 nM (M₅)) at intact CHO-hMₓ cells (x = 1-5). Data analyzed by four-parameter logistic fits, represent mean values ± SEM from at least three independent experiments (performed in triplicate).
### Table 1. MR affinities (K\textsubscript{i} values) of the monomeric xanomeline derivative 41, TBPB derivative 67, DIBA derivative 95, the homodimeric ligands 112 and 113 ('DIBA-DIBA') and 46, 53-55 ('xanomeline-xanomeline'), as well as the heterodimeric ligands 97, 105a, 106, 110 and 111 ('DIBA-xanomeline'), 98, 107, 108, 114 and 115 ('DIBA-TBPB'), 102 and 120 ('DIBA-77-LH-28-1'), 100, 101, 118 and 119 ('DIBA-propantheline') and 99, 116 and 117 ('DIBA-4-DAMP') obtained from equilibrium binding studies with [\textsuperscript{3}H]NMS at live CHO-hMx cells (x = 1-5).

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<th>No.</th>
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<th>M\textsubscript{3}R</th>
<th>M\textsubscript{4}R</th>
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<td>5.8 ± 2.3</td>
<td>-1.5 ± 0.22</td>
<td>100 ± 27</td>
</tr>
<tr>
<td>102</td>
<td>4.7 ± 0.83</td>
<td>0.60 ± 0.13</td>
<td>-1.4 ± 0.22</td>
<td>140 ± 11</td>
</tr>
<tr>
<td>105a</td>
<td>5.2 ± 0.89</td>
<td>0.51 ± 0.06</td>
<td>-2.2 ± 0.05</td>
<td>63 ± 5.5</td>
</tr>
<tr>
<td>106</td>
<td>4.7 ± 0.36</td>
<td>0.46 ± 0.03</td>
<td>-1.1 ± 0.06</td>
<td>140 ± 10</td>
</tr>
<tr>
<td>107</td>
<td>4.9 ± 0.33</td>
<td>0.71 ± 0.11</td>
<td>-1.9 ± 0.05</td>
<td>61 ± 9.0</td>
</tr>
<tr>
<td>108</td>
<td>19 ± 3.1</td>
<td>0.82 ± 0.17</td>
<td>-1.1 ± 0.13</td>
<td>610 ± 120</td>
</tr>
<tr>
<td>110</td>
<td>1.8 ± 0.47</td>
<td>0.26 ± 0.08</td>
<td>-1.8 ± 0.09</td>
<td>19 ± 6.3</td>
</tr>
<tr>
<td>111</td>
<td>1.6 ± 0.29</td>
<td>0.35 ± 0.03</td>
<td>-2.4 ± 0.15</td>
<td>14 ± 0.71</td>
</tr>
<tr>
<td>112</td>
<td>2.5 ± 0.56</td>
<td>0.15 ± 0.02</td>
<td>-1.5 ± 0.12</td>
<td>17 ± 2.0</td>
</tr>
<tr>
<td>113</td>
<td>3.5 ± 0.59</td>
<td>0.58 ± 0.07</td>
<td>-1.3 ± 0.07</td>
<td>13 ± 2.1</td>
</tr>
<tr>
<td>114</td>
<td>1.9 ± 0.37</td>
<td>0.20 ± 0.03</td>
<td>-1.4 ± 0.12</td>
<td>17 ± 1.1</td>
</tr>
<tr>
<td>115</td>
<td>3.3 ± 0.90</td>
<td>0.37 ± 0.05</td>
<td>-1.8 ± 0.15</td>
<td>28 ± 2.4</td>
</tr>
<tr>
<td>116</td>
<td>6.0 ± 0.66</td>
<td>0.84 ± 0.35</td>
<td>-1.6 ± 0.23</td>
<td>77 ± 36</td>
</tr>
<tr>
<td>117</td>
<td>4.8 ± 0.69</td>
<td>1.2 ± 0.19</td>
<td>-2.1 ± 0.16</td>
<td>30 ± 2.9</td>
</tr>
<tr>
<td>118</td>
<td>5.2 ± 1.8</td>
<td>0.80 ± 0.35</td>
<td>-1.5 ± 0.31</td>
<td>29 ± 4.1</td>
</tr>
<tr>
<td>119</td>
<td>3.9 ± 1.3</td>
<td>1.1 ± 0.19</td>
<td>-2.3 ± 0.17</td>
<td>19 ± 2.6</td>
</tr>
<tr>
<td>120</td>
<td>3.6 ± 0.69</td>
<td>0.24 ± 0.02</td>
<td>-1.3 ± 0.13</td>
<td>13 ± 0.76</td>
</tr>
</tbody>
</table>
3.2.3. Functional studies

The heterodimeric dibenzodiazepinone derivatives 106 and 115 were investigated in an IP accumulation assay using HEK-293 cells transiently transfected with the human M₂R and the hybrid G-protein Gαₕ-Gαᵢ₅-HA. Compound 1 caused a concentration-dependent increase in IP₁ accumulation with a pEC₅₀ of 6.93 ± 0.09 (n = 8). By contrast, 106 and 115 did not induce an IP₁ accumulation when studied in agonist mode (cf. Figure 3A), that is, both compounds were not capable of stabilizing a G-protein activating conformation of the M₂R. To allow an estimation of the inhibitory potency of 106 and 115, we tested the ability of compounds 7, 106 and 115 to antagonize the effect of 1 at a concentration corresponding to EC₈₀ (0.3 µM). Compounds 7, 106 and 115 completely inhibited the IP₁ accumulation elicited by 1 proving that these compounds are M₂R antagonists as previously reported for 8³⁴ (cf. Figure 3B).

![Figure 3](image)

**Figure 3.** Investigation of M₂R agonism and antagonism of compounds 106 and 115 in an IP₁ accumulation assay using HEK-hM₂-G₃δ cells. (A) Concentration-dependent effect of 1, 106 and 115 on the accumulation of IP₁. 106 and 115 elicited no response. pEC₅₀ of 1: 6.93 ± 0.09 (mean ± SEM from eight independent experiments performed in triplicate). (B) Concentration-dependent inhibition of the IP₁ accumulation (induced by 1, 0.3 µM) by 7, 106 and 115. Corresponding pKᵦ values: 7: 8.63, 106: 7.16, 115: 7.91. Data represent the means ± SEM from at least five independent experiments (each performed in triplicate).

3.2.4. Synthesis and characterization of the radiolabeled ligands [³H]106 and [³H]115

Aiming at the preparation of the high-affinity propionamides 106 and 115 in their tritiated form, the stabilities of 106 and 115 in aqueous solution were investigated at pH 7.4 in PBS over 48 h. The compounds proved to be stable under these conditions (cf. Figure 4). For the synthesis of [³H]106 and [³H]115 an excess of the precursor amines 105a or 114 was treated with succinimidyl [³H]propionate to afford [³H]106 with a radiochemical yield of 36% and a specific activity of 2.420 TBq/mmol, and [³H]115 with a radiochemical yield of 35% and a specific activity of 1.815 TBq/mmol. The radioligands were obtained in high radiochemical purities (98% and 99%, respectively; cf. Figure 5B and 5D). Quality controls by RP-HPLC after ten months
of storage as solution in ethanol at -20 °C revealed an excellent stability of [³H]115 and approximately 10% decomposition of [³H]106 (cf. Figure 5C and 5E).

Figure 4. HPLC analysis of 106 (A) and 115 (B) after incubation in PBS (pH = 7.4) at 23 °C for up to 48 h. 106 and 115 showed no decomposition. HPLC conditions see experimental section.
Figure 5. Preparation, purity and identity control of the radiolabeled dibenzodiazepinone derivatives \( ^{3}\text{H}\)106 and \( ^{3}\text{H}\)115. (A) Synthesis of \( ^{3}\text{H}\)106 and \( ^{3}\text{H}\)115 by \( ^{3}\text{H}\)propionylation of the amine precursors 105a and 114, respectively, using succinimidyl \( ^{3}\text{H}\)propionate (\( ^{3}\text{H}\)104). (B, C) HPLC analysis of \( ^{3}\text{H}\)106 (B: 0.18 μM, C: 0.17 μM) spiked with “cold” 106 (3 μM), analyzed 3 days after synthesis and after 10 months of storage at -20 °C in EtOH/H\(_2\)O (1:1). (D, E) HPLC analysis of \( ^{3}\text{H}\)115 (D: 0.23 μM, E: 0.23 μM) spiked with “cold” 115 (3 μM), analyzed 3 days after synthesis and after 10 months of storage at -20 °C in EtOH/H\(_2\)O (1:1). HPLC conditions see experimental section.

3.2.5. Characterization of \( ^{3}\text{H}\)106 and \( ^{3}\text{H}\)115

Initially, saturation binding experiments with the tritiated radioligands \( ^{3}\text{H}\)106 and \( ^{3}\text{H}\)115 were performed on intact adherent CHO-hM\(_2\) cells in white-transparent 96-wells plates revealing \( K_d \) values of 1.5 and 0.37 nM, respectively (mean values from at least three independent
experiments performed in triplicate) (cf. Figure 6B and 6D). At concentrations around the $K_d$ value, unspecific binding amounted to approx. 40% ($[^3H]106$) and 30% ($[^3H]115$) of total binding. Saturation binding studies with $[^3H]106$ and $[^3H]115$ performed at CHO-hM$_2$ cell homogenates, precluding the detection of unspecific binding of the radioligand to the microplate, resulted in lower unspecific binding (< 25% of total binding at $K_d$) and yielded binding constants of 1.1 and 0.12 nM, respectively (cf. Figure 6A and 6C). Because of the higher ratio of specific over unspecific binding when using cell homogenates, further studies (association and dissociation kinetics, competition binding experiments and saturation binding of $[^3H]115$ in the presence of the allosteric modulator (15) were performed with CHO-hM$_2$ cell homogenates.

To be noticed, as the orthosteric antagonist 7, used to determine unspecific binding in saturation binding studies, was able to completely prevent one-site (monophasic) specific binding of $[^3H]106$ and $[^3H]115$ to the M$_2$R, these data strongly suggest that the heterodimeric dibenzodiazepinone-type MR ligands 106 and 115 address the orthosteric binding site of the M$_2$R.

Figure 6. Representative saturation isotherms of specific M$_2$R binding (shown in dashed line) of $[^3H]106$ (A, B) and $[^3H]115$ (C, D) obtained from experiments either performed with CHO-hM$_2$ cell homogenates (A, C) or live adherent CHO-hM$_2$ cells (B, D). Non-specific binding (shown in blue) was determined in the presence of the orthostERICally binding MR antagonist atropine (500-fold excess). Experiments were performed in triplicate. Specific binding data were analyzed by an equation describing a one-site binding. Error bars of specific binding and error bars in the Scatchard plots represent propagated errors calculated according to the Gaussian law of errors. Error bars of total and nonspecific binding represent the SEM.

The association of both, $[^3H]106$ and $[^3H]115$, to the M$_2$R was monophasic and resulted in
comparable $k_{on}$ values (cf. Figure 7A and 7C, Table 2). Plateaus were reached after approximately 40 min and 20 min, respectively. With a half-life of 53 min, $[^{3}H]106$ completely dissociated from the $M_2R$. By contrast, the dissociation of $[^{3}H]115$ was incomplete, reaching a plateau at 48% of initially bound radioligand, with a half-life of 32 min (cf. Figure 7B and 7D). This result suggests in part a (pseudo)irreversible (long lasting) binding of $[^{3}H]115$. One reason could be conformational adjustments of the receptor upon ligand binding$^{58}$, another reason might be an enhanced rebinding capability of the dimeric ligand by a simultaneous interaction with two or more binding sites$^{59}$. The kinetically derived dissociation constants $K_d$(kin), calculated according to $K_d$(kin) = $k_{off}$/$k_{on}$, amounted to 0.33 nM for $[^{3}H]106$ and 0.057 nM for $[^{3}H]115$ and were in good agreement with the $K_d$ values obtained from saturation binding experiments ($[^{3}H]106$: $K_d$ = 1.1 nM, $[^{3}H]115$: $K_d$ = 0.12 nM), indicating that both radioligands follow (in part) the law of mass action$^{60}$. An overview of the $M_2R$ binding characteristics of $[^{3}H]106$ and $[^{3}H]115$ is provided in Table 2.

**Figure 7.** Association and dissociation kinetics of $[^{3}H]106$ (A, B) and $[^{3}H]115$ (C, D) determined at CHO-hM2 cell homogenates at 23 °C. (A) Association of $[^{3}H]106$ ($c = 2.0$ nM) to the $M_2R$. Inset: ln[$B_{eq}/(B_{eq} - B_t)$] versus time, $k_{obs}$ = slope = 0.071 min$^{-1}$. (B) Dissociation of $[^{3}H]106$ (preincubation: 4 nM, 1 h) from the $M_2R$ determined in the presence of atropine (1000-fold excess); monophasic exponential decline, $t_{1/2}$ = 53 min. Inset: ln[$B_t/B_0$] versus time. Slope ($-1$) = $k_{off}$ = 0.010 min$^{-1}$. (C) Association of $[^{3}H]115$ ($c = 0.6$ nM) to the $M_2R$. Inset: ln[$B_{eq}/(B_{eq} - B_t)$] versus time, $k_{obs}$ = slope = 0.15 min$^{-1}$. (D) Dissociation of $[^{3}H]115$ (preincubation: 0.6 nM, 1 h) from the $M_2R$ determined in the presence of atropine (1000-fold excess), monophasic exponential decline, $t_{1/2}$ = 32 min. Inset: ln[$B_t/B_0$] versus time. Slope ($-1$) = $k_{off}$ = 0.005 min$^{-1}$. Data represent the mean ± SEM from three (A, B, D) or two (C) independent experiments (each performed in triplicate).
Table 2. M₂R binding characteristics of [³H]106 and [³H]115.

<table>
<thead>
<tr>
<th>radio-ligands</th>
<th>Saturation binding</th>
<th>Binding kinetics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_a$ [nM]$^a$</td>
<td>$K_d$ [nM]$^b$</td>
</tr>
<tr>
<td>[³H]106</td>
<td>1.5 ± 0.26</td>
<td>1.1 ± 0.20</td>
</tr>
<tr>
<td>[³H]115</td>
<td>0.37 ± 0.03</td>
<td>0.12 ± 0.02</td>
</tr>
</tbody>
</table>

$^a$Dissociation constant determined by saturation binding at live CHO-hM₂R cells; mean ± SEM from three independent experiments (performed in triplicate).

$^b$Dissociation constant determined by saturation binding at CHO-hM₂ cell homogenates; mean ± SEM from at least three independent experiments (performed in triplicate).

$^c$Kinetically derived dissociation constant ± propagated error ($K_d$(kin) = $k_{off}$/ $k_{on}$).

$^d$Association rate constant ± propagated error, calculated from $k_{obs}$, $k_{off}$ and the applied radioligand concentration (cf. experimental section).

$^e$Dissociation rate constant and half-life; mean ± SEM from three independent experiments (performed in triplicate).

3.2.6. M₂R equilibrium competition binding with [³H]106 and [³H]115

Figure 8A shows the concentration-dependent effects of the orthosteric MR antagonists 7 and 11 as well as of the allosteric modulator 15 on M₂R equilibrium binding of [³H]106, and Figure 8B presents the concentration-dependent effects of various reported orthosteric (2, 7), allosteric (14, 15, 16) and dualsteric (8, 11) MR ligands as well as 115 on M₂R equilibrium binding of [³H]115. All investigated compounds were capable of totally inhibiting (displacing) specific M₂R binding of [³H]106 or [³H]115, suggesting either a competitive mechanism or a strongly negative cooperativity between the studied compounds and the radiolabeled dibenzodiazepinone derivatives. Generally, $K_i$ values obtained from equilibrium competition binding experiments with [³H]106 and [³H]115 were in good agreement with reported $K_i$ values (cf. Table 3). However, $K_i$ values determined with [³H]106 and [³H]115 were consistently higher than $K_i$ values from competition binding experiments with [³H]NMS (compounds 2, 7, 8, 11, cf. Table 3). The reason for this discrepancy might be due to a multivalent binding mode of the heterodimeric radioligand (reflected by an in part long-lasting binding), therefore, higher competitor concentrations were required for the displacement of [³H]115. Equilibrium competition binding experiments with [³H]115 and 8, as well as with [³H]115 and 11, were also performed after preincubation of CHO-hM₂ cell homogenates with 8 or 11 for 90 min. However, incubation with the competitors prior to the addition of [³H]115 did not result in an increase in the apparent affinities of 8 and 11 (cf. Figure 9).
Figure 8. Concentration-dependent effects of various reported orthosteric (2, 7), dualsteric (8, 11), allosteric (14, 15 and 16) MR ligands and 115 on M2R equilibrium binding of $[^3H]106$ ($c = 2.0 \text{nM}, K_d = 1.1 \text{nM}$) (A) and $[^3H]115$ ($c = 0.3 \text{nM}, K_d = 0.12 \text{nM}$) (B) determined at CHO-hM2 cell homogenates at 22 °C. Data analyzed by four-parameter logistic fits, represent mean values ± SEM from at least three independent experiments (performed in triplicate).

Table 3. Comparison of M2R binding data ($K_i$ or IC$_{50}$ values) of various orthosteric (2, 7), allosteric (14, 15 and 16), dualsteric (8, 11) MR ligands and 115 determined with $[^3H]106$, $[^3H]115$ or $[^3H]$NMS

<table>
<thead>
<tr>
<th>ligand</th>
<th>$[^3H]106$ $K_i$ [nM]</th>
<th>$[^3H]115$ $K_i$ [nM]</th>
<th>$[^3H]$NMS $K^*$ or IC$_{50}^{**}$ [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>-</td>
<td>2300 ± 260</td>
<td>210 ± 59$^{ab}$</td>
</tr>
<tr>
<td>7</td>
<td>2.8 ± 0.60</td>
<td>4.6 ± 1.6</td>
<td>0.94 ± 0.19$^{b}$</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>29 ± 9.6</td>
<td>2.0 ± 0.21$^{b}$</td>
</tr>
<tr>
<td>11</td>
<td>5.2 ± 2.9</td>
<td>6.3 ± 1.3</td>
<td>0.79 ± 0.10$^{b}$</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>3500 ± 570</td>
<td>2200 ± 410$^{bc}$</td>
</tr>
<tr>
<td>15</td>
<td>1800 ± 900</td>
<td>1200 ± 530</td>
<td>460 ± 130$^{bc}$</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>4900 ± 210</td>
<td>&gt;10000$^{ac}$</td>
</tr>
<tr>
<td>115</td>
<td>-</td>
<td>0.34 ± 0.15</td>
<td>0.37 ± 0.05$^{b}$</td>
</tr>
</tbody>
</table>

$a$Determined by equilibrium competition binding with $[^3H]106$ ($c = 2 \text{nM}$) or $[^3H]115$ ($c = 0.3 \text{nM}$) at CHO-hM2 cell homogenates; mean values ± SEM from at least three independent experiments (performed in triplicate). $b$Determined by equilibrium competition binding with $[^3H]$NMS ($c = 0.2 \text{nM}$) at live CHO-hM2 cells; mean ± SEM from at least three independent experiments (performed in triplicate). $c$IC$_{50}$ values obtained from nonlinear four-parameter logistic curve analyses of data characterizing the inhibition of $[^3H]$NMS ($c = 0.2 \text{nM}$) equilibrium binding at live CHO-hM2 cells; mean ± SEM from at least three independent experiments (performed in triplicate).

Figure 9. Concentration-dependent effects of MR ligands 8 (A) and 11 (B) on M2R equilibrium binding of $[^3H]115$ ($c = 0.3 \text{nM}, K_d = 0.12 \text{nM}$) determined at CHO-hM2 cell homogenates at 22 °C, 8 or 11 was preincubated with the M2R for 90 min before the addition of $[^3H]115$. Data analyzed by four-parameter logistic fits, represent mean values ± SEM from three independent experiments (performed in triplicate).
3.2.7. Schild-like analysis with allosteric modulator 15 at the M2R using [3H]115

Competition binding studies with [3H]NMS and 106, and [3H]NMS and 115 at the M2R, as well as M2R saturation binding experiments with [3H]106 and [3H]115 (see above) suggested that the dibenzodiazepinone-type heterodimeric ligands 106 and 115 bind to the orthosteric site of the M2R. Furthermore, competition binding experiments with [3H]106 or [3H]115 at the M2R, for instance with the allosteric modulator 15, resulting in sigmoidal curves which reached 0% specific binding of the dimeric radioligands, indicated binding of [3H]106 and [3H]115 to the allosteric vestibule of the M2R. In order to substantiate the hypothesis of dualsteric binding of dibenzodiazepinone-type heterodimeric ligands such as 115 at the M2R, saturation binding studies with [3H]115 were performed in the presence of the allosteric modulator 15 (cf. Figure 10). This kind of experiment is equivalent to the Schild analysis used to investigate the inhibiting effect of a receptor antagonist on the response elicited by an agonist and is considered the experiment of choice to unveil competitive or non-competitive mechanisms62-65. For instance, it was used to prove the competitive interplay between the allosteric modulator brucine and the pirenzepine derived fluorescent tracer Bo(22)Pz at M1R62.

As becomes obvious from Figure 10A the presence of 15 led to a parallel rightward shift of the saturation isotherms of [3H]115. A plot of log(affinity shift - 1) versus log(concentration of 15) yielded a straight line (‘Schild’ regression, cf. Figure 10B) with a slope not different from unity (slope = 0.97 ± 0.06, P > 0.5), indicating a competitive interaction between [3H]115 and the allosteric modulator 15, and thus suggesting again a dualsteric binding of 115 at the M2R. The ‘pA2’ value (7.05 ± 0.23) derived from the ‘Schild’ regression was in accordance with the pK value (6.06 ± 0.27) from equilibrium competition binding studies with [3H]115 and 15 as well as with reported M2R binding data of 15 (pK 6.00/6.5335).

![Figure 10](image)

Figure 10. Effect of the allosteric modulator 15 on saturation binding of [3H]115 determined at CHO-hM2 cell homogenates at 22 °C. (A) Saturation isotherm of specific radioligand binding plotted in linear and in semi-logarithmic scale. (B) “Schild” regression resulting from the rightward shifts (ΔpKd) of the saturation isotherms (log(r-1) plotted vs. log(concentration 15), where r = 10^(ΔpKd)). The presence of compound 15 led to a parallel rightward shift of the saturation isotherms of [3H]115. The slope of the linear “Schild” regression was not different from unity (P > 0.5, based on the slope mean value ± SEM (0.97 ± 0.06) from three sets of independent saturation binding experiments (performed in triplicate))
indicating a competitive interaction between \([^3\text{H}]115\) and 15. Data represent mean values ± SEM from three independent experiments (each performed in triplicate).

### 3.3. Conclusion

The present study focused on the synthesis and characterization of a series of dibenzodiazepinone-type dimeric MR ligands comprising two homodimeric ligands (‘DIBA-DIBA’) and nineteen heterodimeric ligands (5 × ‘DIBA-xanomeline’, 5 × ‘DIBA-TBPB’, 2 × ‘DIBA-77-LH-28-1’, 4 × ‘DIBA-propantheline’ and 3 × ‘DIBA-4-DAMP’). The most interesting finding was that all of these DIBA-derived dimeric ligands exhibited high M2R affinities (\(K_i\) values: 0.08-5.8 nM, cf. Table 1), i.e. variation of the type of the linker (short vs. long, basic vs. non-basic, etc.) and the second pharmacophoric group had almost no impact on M2R binding. In other words, it was surprising that the bulky ‘side chain’ attached to the dibenzodiazepinone moiety did hardly influence or disturb M2R binding. As the monomeric and dimeric reference compounds (41, 46, 53-55, 67), devoid of the dibenzodiazepinone moiety, exhibited considerably lower M2R affinity compared with the DIBA-derived ligands, the high M2R affinity of the heterodimeric dibenzodiazepinone-type ligands is most likely mediated by the ‘dibenzodiazepinone’ pharmacophore, which presumably binds to the orthosteric site of the M2R. The ‘DIBA-xanomeline’ type heterodimeric ligand 97 displayed the highest M2R affinity (\(K_i\) 0.08 nM) and exhibited the highest M2 subtype preference within the series of presented MR ligands. Two tritium-labeled heterodimeric M2R ligands ([^3\text{H}]106 and [^3\text{H}]115) were prepared and characterized by saturation binding experiments, kinetic studies and equilibrium competition binding with various MR ligands. With a \(K_d\) value of 0.12 nM and high chemical stability, \([^3\text{H}]115\) proved to be an interesting new molecular tool for studying muscarinic receptors and MR ligands. The results from various M2R binding experiments with [^3\text{H}]115, in particular saturation binding studies in the absence or presence of the allosteric MR ligand W84 (15), strongly indicated a simultaneous interaction of 115 with the orthosteric and the ‘common’ allosteric binding site, i.e a dualsteric binding mode at the M2R. This work suggests dibenzodiazepinone-type MR ligands as an interesting compound class to develop highly selective M2R ligands according to the dualsteric (bitopic) ligand approach.

### 3.4. Experimental section

#### 3.4.1. Chemistry

#### 3.4.1.1. General experimental conditions

Commercial reagents and chemicals were purchased from Acros Organics (Geel, Belgium), IRIS Biotech GmbH (Marktredwitz, Germany), Alfa Aesar GmbH & Co. KG (Karlsruhe,
Germany), Merck KGaA (Darmstadt, Germany), Sigma-Aldrich Chemie GmbH (Munich, Germany), TCI Europe (Zwijndrecht, Belgium), MP Biomedicals (Eschwege, Germany), Absource Diagnostic (Munich, Germany) or Abcam (Cambridge, UK) and used without further purification. Technical grade solvents (acetone, ethyl acetate, light petroleum (40-60 °C) and dichloromethane) were distilled before use. Deuterated solvents for NMR spectroscopy were from Deutero GmbH (Kastellaun, Germany). Acetonitrile for HPLC (gradient grade) was obtained from Merck (Darmstadt, Germany). Anhydrous DMF was purchased from Sigma-Aldrich Chemie GmbH. The radiolabeled MR antagonist [3H]NMS (specific activity = 80 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO) via Hartman Analytics GmbH (Braunschweig, Germany). Millipore water was used throughout for the preparation of buffers and HPLC eluents. If moisture-free conditions were required, reactions were performed in dried glassware under inert atmosphere (argon). Anhydrous THF was obtained by distillation over sodium, and anhydrous CH2Cl2 was prepared by distillation over P2O5 after predrying over CaCl2. Reactions were monitored by TLC using aluminum plates coated with silica gel (Merck silica gel 60 F254, thickness 0.2 mm). Spots were detected by UV light (254 nm or 366 nm) or by staining using a 0.3% solution of ninhydrine in n-butanol (amines) or iodine. Flash chromatography was performed in glass columns on silica gel (Merck silica gel 60, 40-63 μm). Polypropylene reaction vessels (1.5 or 2 mL) with screw cap (Süd-Laborbedarf, Gauting, Germany) were used for the synthesis of radioligands ([3H]106 and [3H]115), for small scale reactions, for the investigation of chemical stabilities (106, 115) and for the preparation and storage of stock solutions. All melting points are uncorrected and were measured with a Büchi 530 (Büchi GmbH, Essen, Germany) apparatus. Microwave assisted reactions were performed with an Initiator 2.0 synthesizer (Biotage, Uppsala, Sweden).

NMR spectra were recorded on a Bruker Avance 300 (7.05 T, 1H: 300.1 MHz, 13C: 75.5 MHz), Bruker Avance III HD 400 (9.40 T, 1H: 400 MHz, 13C: 100 MHz) or a Bruker Avance III HD 600 equipped with a cryogenic probe (14.1 T 1H: 600.1 MHz, 13C: 150.9 MHz) (Bruker, Karlsruhe, Germany) with TMS as external standard. Abbreviations for the multiplicities of the signals: s (singlet), d (doublet), t (triplet), dd (doublet of doublet), q (quartet), m (multiplet), brs (broad singlet). For compound 97, 106 and 115, 2D-NMR techniques (1H-COSY, HSQC, HMBC) were used to assign 1H and 13C chemical shifts. IR spectra were measured with a NICOLET 380 FT-IR spectrophotometer (Thermo Electron Corporation). Low-resolution mass spectrometry (MS) was performed on a Finnigan SSQ 710A instrument (CI-MS) (Thermo Finnigan, San Jose, CA). High-resolution mass spectrometry (HRMS) analysis was performed on an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA) using an ESI source. Preparative HPLC was performed on a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. A Kinetex-XB C18, 5 μm, 250 mm × 21 mm (Phenomenex, Aschaffenburg, Germany) served as stationary phase
at a flow-rate of 15 mL/min using mixtures of acetonitrile and 0.1% aq TFA as mobile phase. A detection wavelength of 220 nm was used throughout. Lyophilisation of the collected fractions was performed with an Alpha 2-4 LD apparatus (Martin Christ, Osterode am Harz, Germany) equipped with a RZ 6 rotary vane vacuum pump (Vacuubrand, Wertheim, Germany). Elemental analysis was performed with a Vario MICRO Cube elemental analyzer (Elementar Analysensysteme, Hanau, Germany). Analytical HPLC analysis was performed on a system from Merck-Hitachi (Hitachi, Düsseldorf, Germany) composed of a L-6200-A pump, an AS-2000A autosampler, a L-4000A UV detector, a D-6000 interface. A Kinetex-XB C18, 5 μm, 250 mm × 4.6 mm (Phenomenex, Aschaffenburg, Germany) was used as stationary phase at a flow rate of 0.8 mL/min. Mixtures of acetonitrile (A) and 0.1% aq TFA (B) were used as mobile phase (degassed by Helium purging). The following linear gradient was applied: 0-30 min: A/B 5:95-85:15, 30-32 min: 85:15-95:5, 32-40 min: 95:5. Detection was performed at 220 nm throughout. The oven temperature was 30 °C. The HPLC purity of all analyzed compounds was >95%.

Annotation concerning the NMR spectra (\(^1\)H, \(^{13}\)C) of the dibenzodiazepinone derivatives (92-102, 105a, 106-108 and 110-120): due to a slow rotation about the exocyclic amide group on the NMR time scale, two isomers (ratios provided in the experimental protocols) were evident in the \(^1\)H- and \(^{13}\)C-NMR spectra. The isolated DIBA-type homodimeric ligand 105b was handed to Andrea Pegoli in our research group for further processing. In addition, the number of the TFA salts of the piperazine derivatives was identified by elemental analysis (compounds 97 and 117).

3.4.1.2. Experimental protocols and analytical data

**Ethyl 2-(benzylamino)-2-oxoacetate (21)**

Diethyl oxalate (19) (2.0 g, 13.68 mmol) was mixed in chloroform (100 mL) in a 250-mL three-necked round bottom flask. A solution of benzylamine (20) (1.3 g, 13.68 mmol) in chloroform (50 mL) was added slowly to the reaction mixture. The reaction mixture was refluxed overnight. The solid formed during the reaction was removed by filtration and discarded. The combined filtrate and washings were concentrated under reduced pressure to give compound 21 as yellow oil (1.8 g, 65%). After cooling in the refrigerator (ca. -20 °C) overnight the oil crystallized to form a yellow solid, m.p. 45-48 °C (Lit\(^{43}\) m.p. 50-51 °C). \(R_f=0.2\) (light petroleum/ethyl acetate 6:1 v/v). \(^1\)H-NMR (300 MHz, CDCl\(_3\)): \(\delta\) (ppm) 1.38 (t, J 7.1 Hz, 3H), 4.34 (q, J 7.1 Hz, 2H), 4.52 (d, J 6.0 Hz, 2H), 7.27-7.39 (m, 5H), 7.41 (brs, 1H). \(^{13}\)C-NMR (75 MHz, CDCl\(_3\)): 14.0, 43.9, 63.3, 127.9, 128.0, 128.9, 136.8, 156.5, 160.7. HRMS (ESI): \(m/z\) [M+H]\(^{+}\) calcd. for \([C_{11}H_{14}NO_3]^{+}\) 208.0968, found: 208.0971. C\(_{11}\)H\(_{13}\)NO\(_3\) (207.23).
**N1-Benzyl-N2-methyoxyalamide (22)**

To a solution of compound 21 (1.0 g, 4.83 mmol) in abs. ethanol (20 mL) was added a 2 M solution of methylamine (3.62 mL, 7.24 mmol) in THF. A white solid was formed instantly. After 8 h, collected the solid by filtration, evaporation of the filtrate provided a second portion of product. Combined two portions of product to yield compound 22 as white powder (900 mg, 97%), m.p. 160-163 °C (Lit°. m.p. 184-185 °C), which was used without further purification. 

Rf = 0.3 (light petroleum/ethyl acetate 3:1 v/v).

1H-NMR (300 MHz, CDCl3): δ (ppm) 2.91 (d, J 6.0 Hz, 3H), 4.49 (d, J 6.0 Hz, 2H), 7.25-7.38 (m, 5H), 7.55 (brs, 1H), 7.83 (brs, 1H).


**N1-Benzyl-N2-methylethane-1,2-diamine (23)**

Lithium aluminum hydride (143 mg, 3.77 mmol) was placed in a 50 mL three-necked round bottom flask with abs. THF (15 mL) under an atmosphere of argon. The suspension was immersed in an ice bath and compound 22 (290 mg, 1.51 mmol) dissolved in abs. THF (10 mL) was added to the solution dropwise. The reaction mixture was refluxed overnight. The flask was immersed in an ice bath for quenching, water (0.15 mL), 15% aq NaOH (0.45 mL) and water (0.15 mL) were added dropwise. The suspension was stirred at 0 °C for 30 min. Filtered the white solid, washed the white solid with chloroform (3 x 10 mL). The filtrate was concentrated under reduced pressure to give the crude product, which was subjected to flash column chromatography (eluent: CH2Cl2/MeOH/25% aq NH3 90:3:1 v/v/v) to provide compound 23 as colorless oil (150 mg, 60%). Rf = 0.3 (CH2Cl2/MeOH/25% aq NH3 90:9:1 v/v/v).

1H-NMR (300 MHz, CDCl3): δ (ppm) 1.91 (s, 2H), 2.39 (s, 3H), 2.64-2.71 (m, 2H), 2.72-2.74 (m, 2H), 3.77 (s, 2H), 7.18-7.24 (m, 1H), 7.27-7.29 (m, 4H). 13C-NMR (75 MHz, CDCl3): δ (ppm) 36.3, 48.4, 51.4, 53.9, 126.9, 128.1, 128.4, 140.4. HRMS (ESI): m/z [M+H]+ calcd. for [C10H17N2]⁺ 165.1386, found: 165.1387. C10H16N2 (164.25).

**1- Benzyl-4-methyl-6-nitro-1,4-diazepan-6-yl) methanol (25)**

Compound 23 (4.6 g, 28.04 mmol) and 2-nitroethanol (compound 24) (1985 µL, 27.70 mmol) were dissolved in toluene/ethanol (1:1 v/v) (60 mL). Paraformaldehyde (2.5 g, 83.33 mmol) was added in small portions under stirring, and the suspension was heated to reflux for 6 h. The solvent was evaporated, and the crude product was dissolved in CH2Cl2 (20 mL) and washed with H2O (3 x 20 mL). The organic phase was dried over Na2SO4. The product was purified by flash column chromatography (eluent: light petroleum/ethyl acetate 4:1 to 2:1 v/v) to provide compound 25 as yellow oil (6.9 g, 88%). Rf = 0.3 (light petroleum/ethyl acetate 2:1 v/v).

1H-NMR (300 MHz, CDCl3): δ (ppm) 2.45 (s, 3H), 2.53-2.76 (m, 4H), 2.96-3.16 (m, 2H), 3.42-3.50 (m, 2H), 3.54-3.76 (m, 3H), 3.76-3.88 (m, 2H), 7.22-7.37 (m, 5H). 13C-NMR (75 MHz,
tert-butyl (1-benzyl-4-methyl-1,4-diazepan-6-yl)carbamate (27)

A mixture of compound 26 (4.3 g, 17.25 mmol) was dissolved in 95% ethanol (65 mL), Raney 2800 (slurry in H2O, ca 6 mL) was carefully added to the solution, the suspension was stirred in an autoclave (1 L) under an atmosphere of hydrogen at 10 atm at room temperature overnight. The catalyst was filtered off and the filtrate was concentrated to afford the compound 1-benzyl-4-methyl-1,4-diazepan-6-amine as a brown oily residue (3.7 g, 98%). This material (3.7 g, 16.87 mmol) was dissolved in CHCl3 (50 mL) and di-tert-butyl dicarbonate (4.5 g, 20.64 mmol) in CHCl3 (50 mL) was slowly added to this solution. The mixture was stirred at room temperature overnight. H2O (50 mL) was added followed by extraction with CH2Cl2 (3 x 50 mL). The combined extracts were dried over Na2SO4 and the volatiles were evaporated to afford the crude product, which was subjected to flash column chromatography (eluent: CH2Cl2/MeOH/25% aq NH3 90:3:1 v/v/v/v) to yield compound 27 as yellow oil (2.9 g, 54%). Rf = 0.8 (CH2Cl2/MeOH/25% aq NH3 90:9:1 v/v/v/v). 1H-NMR (300 MHz, CDCl3): δ (ppm) 1.40 (s, 9H), 2.35 (s, 3H), 2.39-2.69 (m, 5H), 2.71-2.91 (m, 3H), 3.55 (d, J 13 Hz, 1H), 3.67 (d, J 13 Hz, 1H), 3.72-3.79 (m, 1H), 5.50 (br. s, 1H), 7.18-7.39 (m, 5H). 13C-NMR (75 MHz, CDCl3): δ (ppm) 28.5, 48.2, 48.8, 56.5, 58.9, 59.5, 62.2, 63.5, 78.9, 127.2, 128.4, 128.9, 139.3, 155.4. HRMS (ESI): m/z [M+H]+ calcd. for [C18H30N3O2]+ 320.2333, found: 320.2342. C18H30N3O2 (319.45).
Compound 27 (200 mg, 0.626 mmol) was suspended in THF/H$_2$O (1:4 v/v) (5 mL) followed by the addition of 10% Pd/C (40 mg). The mixture was stirred in an autoclave (1 L) under an atmosphere of hydrogen at 10 atm at room temperature overnight. Filtered the reaction mixture through a pad of celite, the filtrate was concentrated under reduced pressure to give compound 28 as colorless oil (110 mg, 77%), which was used without further purification. R$_f$ = 0.2 (CH$_2$Cl$_2$/MeOH/25% aq NH$_3$ 90:9:1 v/v/v). $^1$H-NMR (300 MHz, [D$_6$]DMSO): δ (ppm) 1.36 (s, 9H), 2.27 (s, 3H), 2.35-2.49 (m, 4H), 2.58-2.69 (m, 2H), 2.59-2.68 (m, 2H), 2.85-2.91 (m, 1H), 3.49-3.66 (m, 1H), 6.58 (brs, 1H). $^{13}$C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 28.3, 47.2, 49.1, 50.7, 52.7, 60.4, 61.3, 77.7, 154.9. HRMS (ESI): m/z [M+H]$^+$ calcd. for [C$_{11}$H$_{24}$N$_3$O$_2$]$^+$ 230.1863, found: 230.1868. C$_{11}$H$_{23}$N$_3$O$_2$ (229.32).

tert-Butyl (2-bromoethyl)carbamate (30)

2-bromoethan-1-amine hydrobromide (compound 29) (3.0 g, 14.63 mmol) and di-tert-butyl dicarbonate (3.2 g, 14.67 mmol) were dissolved in CH$_2$Cl$_2$ (80 mL). Triethylamine (2.05 mL, 14.71 mmol) was added dropwise and the reaction mixture was stirred at room temperature overnight. CH$_2$Cl$_2$ (20 mL) was added, the mixture was washed with brine, and the organic phase was dried over Na$_2$SO$_4$ followed by removal of the solvent under reduced pressure. The product was purified by flash column chromatography (eluent: light petroleum/ethyl acetate 8:2 v/v) to give compound 30 as yellow oil (2.6 g, 80%). R$_f$ = 0.7 (light petroleum/ethyl acetate 2:1 v/v). $^1$H-NMR (400 MHz, CDCl$_3$): δ (ppm) 1.44 (s, 9H), 3.44 (t, J 5.5 Hz, 2H), 3.47-3.57 (m, 2H), 4.98 (s, 1H). $^{13}$C-NMR (100 MHz, CDCl$_3$): 28.4, 32.8, 42.4, 79.8, 155.5. C$_7$H$_{14}$BrNO$_2$ (224.10).

tert-Butyl (2-(piperazin-1-yl)ethyl)carbamate (32)

Compound 30 (1.0 g, 4.46 mmol), piperazine (compound 31) (1.5 g, 17.44 mmol) and K$_2$CO$_3$ (1.2 g, 8.70 mmol) were added to MeCN (50 mL) and the mixture was kept under reflux for 3 h. The mixture was filtered and the filtrate was concentrated to afford a yellow oily residue, which was dissolved in CH$_2$Cl$_2$ (20 mL) followed by washing with water. The aqueous phase was treated with CH$_2$Cl$_2$ (3 × 20 mL) and the organic extracts were collected. All organic phases were combined and dried over Na$_2$SO$_4$. Removal of the volatiles under reduced pressure gave the crude product, which was subjected to flash column chromatography (eluent: CH$_2$Cl$_2$/MeOH/25% aq NH$_3$ 90:3:1 v/v/v) to yield compound 32 as yellow oil (0.93 g, 91%). R$_f$ = 0.4 (CH$_2$Cl$_2$/MeOH/25% aq NH$_3$ 90:9:1 v/v/v). $^1$H-NMR (300 MHz, CDCl$_3$): δ (ppm) 1.45 (s, 9H), 2.42-2.46 (m, 6H), 2.57 (brs, 1H), 2.83-3.01 (m, 4H), 3.19-3.27 (m, 2H), 4.97 (brs, 1H). $^{13}$C-NMR (75 MHz, CDCl$_3$): 28.5, 36.9, 45.7, 53.6, 57.7, 82.6, 160.0. HRMS (ESI): m/z [M+H]$^+$ calcd. for [C$_{11}$H$_{24}$N$_3$O$_2$]$^+$ 230.1863, found: 230.1869. C$_{11}$H$_{23}$N$_3$O$_2$ (229.32).
2-Amino-2-(pyridin-3-yl)acetonitrile (34)\(^{47}\)

To a cooled (5 °C) solution of potassium cyanide (10.4 g, 159.7 mmol) in water (100 mL) was added 3-pyridinecarbaldehyde (compound 33) (11.4 g, 106.5 mmol) dropwise. Afterwards, acetic acid (9.1 mL, 159.7 mmol) was added over a period of 30 min. The mixture was stirred at room temperature for 2 h followed by extraction with ethyl acetate (3 × 50 mL). The combined organic phases were dried over Na\(_2\)SO\(_4\) and the solvent was removed under reduced pressure to give the intermediate 2-hydroxy-2-(pyridin-3-yl)acetonitrile as yellow solid (14.0 g, 98%), which was used without further purification. \(R_f = 0.4\) (CH\(_2\)Cl\(_2\)/MeOH 10:1 v/v).

\(^1\)H-NMR (300 MHz, CDCl\(_3\)): \(\delta\) (ppm) 5.65 (s, 1H), 7.38 (dd, \(J\) 7.9, 4.9 Hz, 1H), 7.93 (d, \(J\) 9.5 Hz, 1H), 8.45 (dd, \(J\) 4.9, 1.3 Hz, 1H), 8.58 (d, \(J\) 1.9 Hz, 1H). \(^{13}\)C-NMR (75 MHz, CDCl\(_3\)) \(\delta\) (ppm) 60.7, 118.9, 124.6, 133.2, 135.7, 146.8, 149.4. The intermediate (14.0 g, 104.4 mmol) was added to a solution of NH\(_4\)Cl (33.9 g, 633.7 mmol) in H\(_2\)O (100 mL) followed by the addition of 25% aq NH\(_4\)OH (10 mL). The mixture was stirred at room temperature for 20 h. Extracted the mixture with ethyl acetate (10 × 30 mL), the combined organic phases were dried over Na\(_2\)SO\(_4\). Removal of the volatiles under reduced pressure gave compound 34 as brown oil (9.3 g, 67%). \(R_f = 0.3\) (CH\(_2\)Cl\(_2\)/MeOH 10:1 v/v). \(^1\)H-NMR (300 MHz, CDCl\(_3\)): \(\delta\) (ppm) 2.09 (brs, 2H), 4.91 (s, 1H), 7.24-7.34 (m, 1H), 7.78-7.87 (m, 1H), 8.54 (dd, \(J\) 4.8, 1.5 Hz, 1H), 8.65-8.77 (m, 1H). \(^{13}\)C-NMR (75 MHz, CDCl\(_3\)): \(\delta\) (ppm) 45.2, 120.2, 123.8, 132.2, 134.5, 148.3, 150.3. HRMS (ESI): \(m/z\ [M+H]^+\) calcd. for [C\(_7\)H\(_8\)N\(_3\)]\(^+\) 134.0713, found: 134.0713. C\(_7\)H\(_8\)N\(_3\) (133.15).

3-Chloro-4-(pyridin-3-yl)-1,2,5-thiadiazole (35)\(^{47}\)

To a cooled (5-10 °C) solution of S\(_2\)Cl\(_2\) (10.8 mL, 137.2 mmol) in DMF (50 mL) was added a solution of compound 34 (9.1 g, 68.34 mmol) in DMF (65 mL) over a period of 1 h. The mixture was stirred at 5-10 °C for additional 45 min and ice water (30 mL) was added. The formed precipitate was removed by filtration. To the filtrate was added 20% NaOH solution to adjust a pH of 8, thereby keeping the temperature below 20 °C. Extracted the mixture with ethyl acetate (3 × 20 mL). The combined organic phases were dried over Na\(_2\)SO\(_4\) and the volatiles were removed under reduced pressure to give the crude product, which was subjected to flash column chromatography (eluent: light petroleum/ethyl acetate 3:2 v/v) to afford compound 35 as a white solid (9.4 g, 69%), m.p. 40-42 °C (Lit\(^{47}\). m.p. 48-49 °C). \(R_f = 0.7\) (light petroleum/acetone 1:1 v/v). \(^1\)H-NMR (300 MHz, CDCl\(_3\)): \(\delta\) (ppm) 7.41-7.46 (m, 1H), 8.24-8.28 (m, 1H), 8.72 (dd, \(J\) 4.9, 1.6 Hz, 1H), 9.20 (dd, \(J\) 2.2, 0.6 Hz, 1H). \(^{13}\)C-NMR (75 MHz, CDCl\(_3\)): \(\delta\) (ppm) 123.4, 126.9, 135.7, 143.6, 149.4, 150.9, 155.2. HRMS (ESI): \(m/z\ [M+H]^+\) calcd. for [C\(_7\)H\(_8\)ClN\(_3\)S]\(^+\) 197.9887, found: 197.9893. C\(_7\)H\(_8\)ClN\(_3\)S (197.64).

3-((4-(Pyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)propan-1-ol (37)\(^{50}\)

A suspension of 60% NaH in mineral oil (363 mg, 9.47 mmol) was added to abs. THF (10 mL)
under an atmosphere of argon. The mixture was cooled to 0 °C and propane-1,3-diol (compound 36) (460 mg, 6.04 mmol) was added under stirring. The mixture was then kept under reflux for 1 h. A solution of compound 35 (600 mg, 3.03 mmol) in abs. THF (10 mL) was added and reflux was continued for 8 h. The solvent was removed under reduced pressure and ice-cold water was added dropwise to the residue. Extracted the mixture with ethyl acetate (3 × 20 mL). The combined organic phases were dried over Na₂SO₄ and concentrated under reduced pressure to give the crude product, which was subjected to flash column chromatography (eluent: light petroleum/acetone 2:1 v/v) to afford compound 37 as yellow oil (370 mg, 52%). \( R_f = 0.3 \) (light petroleum/acetone 2:1 v/v).

**1H-NMR** (300 MHz, CDCl₃): \( \delta \) (ppm) 2.08-2.20 (m, 2H), 2.19 (brs, 1H), 3.87 (t, \( J = 6.0 \) Hz, 2H), 4.69 (t, \( J = 6.1 \) Hz, 2H), 7.36-7.40 (m, 1H), 8.30-8.54 (m, 1H), 8.62 (dd, \( J = 4.8, 1.6 \) Hz, 1H), 9.36 (dd, \( J = 2.1, 0.7 \) Hz, 1H).

**13C-NMR** (75 MHz, CDCl₃): \( \delta \) (ppm) 31.9, 59.0, 68.2, 123.6, 127.7, 134.9, 144.8, 148.4, 149.9, 162.7.

**HRMS (ESI):** m/z [M+H⁺] calcd. for \([C_{10}H_{12}N_3O_2S]⁺ \) 238.0645, found: 238.0651. \( C_{10}H_{11}N_3O_2S \) (237.28).

3-((4-(1-Methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)propan-1-ol (38)**

To a solution of compound 37 (370 mg, 1.56 mmol) in acetone (5 mL) was added methyliodide (0.97 mL, 15.6 mmol) and the mixture was stirred at room temperature for 24 h. The formed precipitate was collected by filtration and washed with acetone (5 mL). Drying **in vacuo** gave the N-methylated, but non-reduced intermediate as yellow solid (480 mg, 81%). \( R_f = 0.1 \) (CH₂Cl₂/MeOH 10:1 v/v).

**1H-NMR** (300 MHz, [D₄]MeOH): \( \delta \) (ppm) 1.99-2.06 (m, 2H), 3.59-3.65 (m, 2H), 4.45 (s, 3H), 4.62 (t, \( J = 6.3 \) Hz, 2H), 8.28 (dd, \( J = 8.2, 6.2 \) Hz, 1H), 9.07 (dd, \( J = 12, 7.3 \) Hz, 2H), 9.54 (s, 1H). The intermediate (470 mg, 1.24 mmol) was dissolved in MeOH (10 mL). The solution was cooled to -5 °C and NaBH₄ (143 mg, 3.76 mmol) was added carefully. The mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. The residue was dissolved in CH₂Cl₂ (10 mL) followed by washing with water. The aqueous phase was treated with CH₂Cl₂ (3 × 10 mL) and the organic extracts were collected. All organic phases were combined and dried over Na₂SO₄. Removal of the volatiles reduced pressure gave the crude product, which was subjected to flash column chromatography (eluent: CH₂Cl₂/MeOH/25% aq NH₃ 90:9:1 v/v/v) to afford compound 38 as brown oil (130 mg, 41%). \( R_f = 0.3 \) (CH₂Cl₂/MeOH/25% aq NH₃ 85:15:1 v/v/v).

**1H-NMR** (300 MHz, CDCl₃): \( \delta \) (ppm) 2.03-2.11 (m, 2H), 2.38-2.51 (m, 5H), 2.56 (bres, 1H), 2.59 (t, \( J = 5.6 \) Hz, 2H), 3.40-3.54 (m, 2H), 3.78 (t, \( J = 6.1 \) Hz, 2H), 4.59 (t, \( J = 6.1 \) Hz, 2H), 6.87-7.13 (m, 1H).

**13C-NMR** (75 MHz, CDCl₃): \( \delta \) (ppm) 26.5, 32.0, 45.9, 51.2, 54.9, 59.3, 67.9, 128.4, 129.2, 146.7, 162.5. **HRMS (ESI):** m/z [M+H⁺] calcd. for \([C_{11}H_{17}N_3O_2S]⁺ \) 256.1114, found: 256.1115. \( C_{11}H_{17}N_3O_2S \) (257.34).
3-(3-Bromopropoxy)-4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazole (39)\textsuperscript{50}

Compound 38 (400 mg, 1.57 mmol) and PPh\textsubscript{3} (1.2 g, 4.57 mmol) were dissolved in CH\textsubscript{2}Cl\textsubscript{2} (30 mL) and the solution was cooled to -5 \textdegree C under an atmosphere of argon. A solution of CBr\textsubscript{4} (3.4 g, 10.25 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (20 mL) was slowly dropped into the stirred mixture, thereby keeping the temperature of the mixture below 5 \textdegree C. After completed addition, stirring was continued at room temperature for 24 h. The solvent was removed under reduced pressure to give a brown residue, and subjected to flash column chromatography (eluent: light petroleum/acetone/25% aq NH\textsubscript{3} 85:15:1 v/v/v) to afford compound 39 as a brown oil (300 mg, 50%). R\textsubscript{r} = 0.6 (light petroleum/acetone/25% aq NH\textsubscript{3} 65:35:1 v/v/v). \textsuperscript{1}H-NMR (300 MHz, CDCl\textsubscript{3}): \(\delta\) (ppm) 2.26-2.36 (m, 2H), 2.41-2.49 (m, 5H), 2.58 (t, J 5.8 Hz, 2H), 3.45 (dd, J 4.4, 2.5 Hz, 2H), 3.71 (t, J 6.4 Hz, 2H), 4.61 (t, J 6.0, 2H), 6.91-7.08 (m, 1H). \textsuperscript{13}C-NMR (75 MHz, CDCl\textsubscript{3}): \(\delta\) (ppm) 26.5, 29.4, 31.8, 45.8, 51.1, 54.9, 68.4, 128.4, 129.1, 146.7, 161.9. HRMS (ESI): \(m/z\) [M+H]\textsuperscript{+} calcd. for [C\textsubscript{11}H\textsubscript{17}BrN\textsubscript{3}OS]\textsuperscript{*} 318.0270, found: 318.0271. C\textsubscript{11}H\textsubscript{16}BrN\textsubscript{3}OS (318.23).

3-(1-Methyl-1,2,5,6-tetrahydropyridin-3-yl)-4-(3-(piperazin-1-yl)propoxy)-1,2,5 thia diazole (40)

Compound 39 (600 mg, 1.89 mmol) and piperazine (1.3 g, 15.09 mmol) were suspended in MeCN (12 mL) followed by the addition of potassium carbonate (523 mg, 3.78 mmol). The mixture was refluxed for 2 h. Insoluble material was separated by filtration and washed with CH\textsubscript{2}Cl\textsubscript{2} (2 \times 10 mL). The filtrate and washings were combined and the volatiles were evaporated to yield a brown oil-like residue, which was dissolved in CH\textsubscript{2}Cl\textsubscript{2} (20 mL) followed by washing with brine. The aqueous phase was treated with CH\textsubscript{2}Cl\textsubscript{2} (3 \times 10 mL) and the organic extracts were collected. All organic phases were combined and dried over Na\textsubscript{2}SO\textsubscript{4}. The solvent was removed under reduced pressure to yield the crude product, which was subjected to column chromatography (eluent: CH\textsubscript{2}Cl\textsubscript{2}/MeOH/25% aq NH\textsubscript{3} 90:6:1 v/v/v) to obtain compound 40 as yellow oil (405 mg, 66%). R\textsubscript{r} = 0.4 (CH\textsubscript{2}Cl\textsubscript{2}/MeOH/25% aq NH\textsubscript{3} 90:9:1 v/v/v). \textsuperscript{1}H-NMR (300 MHz, [D\textsubscript{4}]MeOH): \(\delta\) (ppm) 1.92-2.13 (m, 2H), 2.30-2.43 (m, 4H), 2.44 (s, 3H), 2.45-2.52 (m, 4H), 2.55 (t, J 5.7 Hz, 2H), 2.90 (t, J 4.7 Hz, 4H), 3.43 (s, 2H), 4.49 (t, J 6.4 Hz, 2H), 7.02-7.04 (m, 1H). \textsuperscript{13}C-NMR (75 MHz, [D\textsubscript{4}]MeOH): \(\delta\) (ppm) 26.2, 26.6, 45.90, 45.92, 51.2, 54.3, 55.0, 55.7, 69.2, 128.3, 129.3, 146.8, 162.4. HRMS (ESI): \(m/z\) [M+H]\textsuperscript{+} calcd. for [C\textsubscript{15}H\textsubscript{26}N\textsubscript{3}OS]\textsuperscript{*} 324.1853, found: 324.1854. C\textsubscript{15}H\textsubscript{25}N\textsubscript{3}OS (323.46).

1-Methyl-4-(3-(4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)propyl)-1,4-diazepan-6-amine tetrakis(hydrotrifluoroacetate) (41)

Compound 39 (490 mg, 1.52 mmol) and compound 28 (354 mg, 1.54 mmol) were suspended in MeCN (20 mL) followed by the addition of potassium carbonate (427 mg, 3.09 mmol). The mixture was stirred at 110 \textdegree C under microwave irradiation for 30 min. Solids were separated...
by filtration and washed with CH₂Cl₂ (2 × 10 mL). The combined filtrate and washings were concentrated under reduced pressure yielding a yellow oily residue, which was dissolved in CH₂Cl₂ (10 mL) followed by washing with brine. The aqueous phase was treated with CH₂Cl₂ (3 × 10 mL) and the organic extracts were collected. All organic phases were combined and dried over Na₂SO₄. Removal of the volatiles under reduced pressure yielded the Boc-protected intermediate as yellow oily residue, which was dissolved in CH₂Cl₂/TFA (4:1 v/v) (5 mL). The mixture was stirred at room temperature overnight. CH₂Cl₂ (10 mL) was added, the volatiles were evaporated and the residue was subjected to purification by preparative HPLC (column: Kinetex XB-C18 5 μm 250 × 21 mm; gradient: 0-35% MeCN in 2 min, 35-66% MeCN in 66 min; detection: 220 nm): 97% (tᵣ = 10.7 min, k = 2.7). HRMS (ESI): m/z [M+H]+ calcd. for [C₁₇H₃₁N₆OS]^+ 367.2275, found: 367.2273. C₁₇H₃₀N₆OS · C₈H₄F₁₂O₈ (366.53 + 456.09).

5-((4-(Pyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)pentan-1-ol (43)
A suspension of 60% NaH in mineral oil (908 mg, 23.69 mmol) was added to abs. THF (40 mL) under an atmosphere of argon. The suspension was cooled to 0 °C, 1,5-pentanediol (compound 42) (2.0 g, 19.20 mmol) was added under stirring, and the mixture was refluxed for 1 h. A solution of compound 35 (1.5 g, 7.58 mmol) in abs. THF (10 mL) was added and reflux was continued for 8 h. The solvent was removed under reduced pressure and ice-cold water was added dropwise to the residue. Extracted the mixture with ethyl acetate (3 × 30 mL), the combined organic phases were washed over Na₂SO₄ and concentrated under reduced pressure to give the crude product, which was subjected to flash column chromatography (eluent: light petroleum/acetone 2:1 v/v) to afford compound 43 as colorless oil (960 mg, 48%). Rᵣ = 0.3 (light petroleum/acetone 2:1 v/v). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 1.50-1.76 (m, 4H), 1.86 (brs, 1H), 1.89-1.98 (m, 2H), 3.69 (t, J 6.2 Hz, 2H), 4.54 (t, J 6.5 Hz, 2H), 7.40 (dd, J 8.0, 4.9 Hz, 1H), 8.42 (d, J 8.0 Hz, 1H), 8.64 (d, J 4.8 Hz, 1H), 9.40 (s, 1H). ¹³C-NMR (75 MHz, CDCl₃): δ (ppm) 21.6, 28.2, 32.6, 62.2, 71.4, 123.4, 127.4, 134.7, 144.5, 147.9, 150.4, 162.7. HRMS (ESI): m/z [M+H]+ calcd. for [C₁₂H₁₈N₃O₂S]^+ 266.0958, found: 266.0966. C₁₂H₁₅N₃O₂S (265.33).

5-((4-(1-Methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)pentan-1-ol (44)
To a solution of compound 43 (0.96 g, 3.62 mmol) in acetone (15 mL) was added methyliodide (2.3 mL, 36.2 mmol) and the mixture was stirred at room temperature for 24 h. The formed precipitate was collected by filtration, washed with acetone and dried under vacuum to yield the N-methylated, but non-reduced intermediate as yellow solid (1.4 g, 95%). Rf = 0.1 (CH2Cl2/MeOH 6:1 v/v). 1H-NMR (300 MHz, [D6]MeOH): δ (ppm) 1.51-1.73 (m, 4H), 1.87-2.07 (m, 2H), 3.60 (t, J 5.9 Hz, 2H), 4.52 (s, 3H), 4.65 (t, J 6.5 Hz, 2H), 8.22 (dd, J 8.1, 6.2 Hz, 1H), 8.96 (d, J 6.1 Hz, 1H), 9.24 (d, J 8.3 Hz, 1H), 9.57 (s, 1H). The intermediate (1.4 g, 3.44 mmol) was dissolved in MeOH (20 mL) and the solution was cooled to -5 °C. NaBH4 (519 mg, 13.66 mmol) was added carefully. The mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. The residue was dissolved in CH2Cl2 (20 mL) followed by washing with water. The aqueous phase was treated with CH2Cl2 (3 × 20 mL) and the organic extracts were collected. All organic phases were combined and dried over Na2SO4. Removal of the volatiles under reduced pressure gave the crude product, which was subjected to flash column chromatography (eluent: CH2Cl2/MeOH/25% aq NH3 97:3:1 v/v/v) to afford compound 44 as brown oil (900 mg, 92%). Rf = 0.3 (CH2Cl2/MeOH/25% aq NH3 85:15:1 v/v/v). 1H-NMR (300 MHz, CDCl3): δ (ppm) 1.47-1.59 (m, 2H), 1.59-1.69 (m, 2H), 1.78-1.98 (m, 2H), 2.39-2.49 (m, 5H), 2.57 (t, J 5.6 Hz, 2H), 3.44 (dd, J 4.4, 2.4 Hz, 2H), 3.66 (t, J 6.3 Hz, 2H), 4.44 (t, J 6.6 Hz, 2H), 7.00-7.09 (m, 1H). 13C-NMR (75 MHz, CDCl3): δ (ppm) 22.3, 26.6, 28.6, 32.3, 45.9, 51.2, 54.9, 62.6, 70.8, 128.4, 129.2, 146.8, 162.5. HRMS (ESI): m/z [M+H]+ calcd. for [C13H22N3O2S]+ 284.1427, found: 284.1430. C13H21N3O2S (283.39).

3-((5-Bromopentyl)oxy)-4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazole (45)

Compound 44 (850 mg, 3.0 mmol) and PPh3 (2.4 g, 9.15 mmol) were dissolved in CH2Cl2 (30 mL) and the solution was cooled to -5 °C under an atmosphere of argon. A solution of CBr4 (6.5 g, 19.59 mmol) in CH2Cl2 (15 mL) was slowly dropped into the stirred mixture, thereby keeping the temperature of the mixture below 5 °C. Stirring was continued at room temperature for 24 h. The solvent was removed under reduced pressure and the residue subjected to column chromatography (eluent: light petroleum/acetone/25% aq NH3 65:35:1 v/v/v) to afford compound 45 as brown oil (740 mg, 71%). Rf = 0.4 (light petroleum/acetone/25% aq NH3 65:35:1 v/v/v). 1H-NMR (300 MHz, CDCl3): δ (ppm) 1.56-1.71 (m, 2H), 1.81-2.01 (m, 4H), 2.39-2.50 (m, 5H), 2.57 (t, J 5.5 Hz, 2H), 3.36-3.52 (m, 4H), 4.46 (t, J 6.4 Hz, 2H), 6.97-7.13 (m, 1H). 13C-NMR (75 MHz, CDCl3): δ (ppm) 24.7, 26.7, 28.0, 32.2, 33.5, 45.9, 51.2, 55.0, 70.4, 128.5, 129.3, 146.8, 162.3. HRMS (ESI): m/z [M+H]+ calcd. for [C13H21BrN3OS]+ 346.0583, found: 346.0585. C13H20BrN3OS (346.29).

1,4-Bis(5-((4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)pentyl)piperazine (46)
Compound 45 (730 mg, 2.11 mmol), potassium carbonate (193 mg, 1.39 mmol), and piperazine (60 mg, 0.70 mmol) were added to MeCN (5 mL). The mixture was stirred at 110 °C under microwave irradiation for 30 min, and cooled to room temperature. Insoluble material was removed by filtration and the filtrate was concentrated under reduced pressure to give the crude product, which was dissolved in CH₂Cl₂ (10 mL) followed by washing with H₂O (3 x 10 mL). The organic phase was dried over Na₂SO₄ and the solvent was removed under reduced pressure. The product was purified by flash column chromatography (eluent: CH₂Cl₂/MeOH/25% aq NH₃ 97:3:1 v/v/v) to afford compound 46 as white solid (96 mg, 22%), m.p. 41-42 °C. Rₜ = 0.7 (CH₂Cl₂/MeOH/25% aq NH₃ 90:9:1 v/v/v). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 1.31-1.61 (m, 8H), 1.71-1.90 (m, 4H), 2.26-2.33 (m, 5H), 2.34-2.38 (m, 4H), 2.39 (s, 6H), 2.41-2.52 (m, 11H), 3.38 (dd, J 4.3, 2.4 Hz, 4H), 4.38 (t, J 6.5 Hz, 4H), 6.85-7.15 (m, 2H). ¹³C-NMR (75 MHz, CDCl₃): δ (ppm) 24.0, 26.5, 26.6, 28.8, 45.9, 51.1, 53.1, 55.0, 58.5, 70.7, 128.3, 129.3, 146.8, 162.5. RP-HPLC (220 nm): 97% (tᵣ = 13.9 min, k = 3.8). HRMS (ESI): m/z [M+H]⁺ calcd. for [C₃₀H₄₈N₉O₅S₂]⁺ 617.3414, found: 617.3407. C₃₀H₄₈N₉O₅S₂ (616.89).

tert-Butyl (5-((4-(pyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)pentyl)carbamate (48)
To a cooled (0 °C) solution of 5-amino-1-pentanol (1.0 g, 9.67 mmol) and triethylamine (1.2 mL, 8.89 mmol) in CH₂Cl₂ (50 mL) was slowly added di-tert-butyl dicarbonate (1.9 g, 8.71 mmol) in CH₂Cl₂ (20 mL). The mixture was stirred at 0 °C for 30 min and stirring was continued at room temperature for additional 12 h. Saturated aq NH₄Cl (20 mL) was added followed by extraction with CH₂Cl₂ (3 x 20 mL). The combined organic extracts was washed with brine, dried over Na₂SO₄ and concentrated under reduced pressure to give the intermediate tert-butyl (5-hydroxypentyl)carbamate (compound 47) as colorless oil (1.9 g, 97%) without purification. Rₜ = 0.5 (light petroleum/acetone 2:1 v/v). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 1.42 (s, 9H), 1.46-1.61 (m, 6H), 1.63 (brs, 1H), 3.12 (t, J 6.9 Hz, 2H), 3.64 (t, J 6.4 Hz, 2H), 4.53 (brs, 1H). ¹³C-NMR (75 MHz, CDCl₃): δ (ppm) 22.9, 28.4, 29.8, 32.2, 40.4, 62.5, 79.2, 156.0. HRMS (ESI): m/z [M+H]⁺ calcd. for [C₁₀H₂₂N₂O₃]⁺ 204.1594, found: 204.1595. To a stirred and cooled (0 °C) solution of compound 47 (307 mg, 1.51 mmol) in abs. THF (5 mL) was added the suspension of 60% NaH in mineral oil (73 mg, 1.90 mmol) in portions under an atmosphere of argon, followed by the addition of 35 (200 mg, 1.01 mmol) dissolved in abs. THF (2 mL). The mixture was stirred at 0 °C for 5 min and slowly warmed up until reflux. Reflux was continued for 2 h. The solvent was removed under reduced pressure and ice-cold water was added dropwise to the residue. Extracted the mixture with ethyl acetate (3 x 5 mL), the combined organic phases were dried over Na₂SO₄ and concentrated under reduced pressure to give the crude product, which was subjected to flash column chromatography (eluent: light petroleum/acetone 5:1 v/v) to afford compound 48 as colorless oil (100 mg, 27%). Rₜ = 0.4 (light petroleum/acetone 2:1 v/v). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 1.43 (s, 9H), 1.47-1.67
(m, 4H), 1.82-1.99 (m, 2H), 3.12-3.18 (m, 2H), 4.52 (t, J 6.5 Hz, 2H), 4.70 (brs, 1H), 7.33-7.51 (m, 1H), 8.33-8.51 (m, 1H), 8.65 (dd, J 4.8, 1.5 Hz, 1H), 9.39 (d, J 1.6 Hz, 1H). 13C-NMR (75 MHz, CDCl3): δ (ppm) 23.3, 28.4, 28.6, 29.8, 40.4, 71.1, 79.1, 123.5, 127.6, 134.7, 144.9, 148.5, 150.1, 156.0, 162.7. HRMS (ESI): m/z [M+H]+ calcd. for [C17H25N4O3S]+ 365.1642, found: 365.1644. C17H20N4O3S (364.46).

tert-Butyl (5-(((4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)pentyl)carbamate (49)

To a solution of compound 48 (3.3 g, 9.05 mmol) in acetone (10 mL) was added methyl iodide (5.7 mL, 91.2 mmol) and the mixture was stirred at room temperature for 24 h. The formed precipitate was collected, washed with acetone and dried under vacuum to afford the N-methylated, but non-reduced intermediate as yellow solid (3.3 g, 96%). \( R_f = 0.1 \) (CH2Cl2/MeOH 6:1 v/v). This intermediate (3.0 g, 7.91 mmol) was dissolved in MeOH (50 mL) and the solution was cooled to -5 °C followed by the careful addition of NaBH4 (2.1 g, 55.26 mmol). The mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure. The residue was dissolved in CH2Cl2 (20 mL) followed by washing with brine. The aqueous phase was treated with CH2Cl2 (3 × 20 mL) and the organic extracts were collected. All organic phases were combined and dried over Na2SO4. Removal of the solvent under reduced pressure gave the crude product, which was subjected to flash column chromatography (eluent: CH2Cl2/MeOH/25%aq NH3 97:3:1 v/v/v) to afford compound 49 as brown oil (2.4 g, 79%). \( R_f = 0.7 \) (CH2Cl2/MeOH/25%aq NH3 90:9:1 v/v/v). 1H-NMR (300 MHz, CDCl3): δ (ppm) 1.37 (s, 9H), 1.38-1.62 (m, 4H), 1.76-1.94 (m, 2H), 2.49 (s, 3H), 2.41-2.52 (m, 2H), 2.60 (t, J 5.6 Hz, 2H), 3.14 (dd, J 13, 6.3 Hz, 2H), 3.46-3.48 (m, 2H), 4.44 (t, J 6.6 Hz, 2H), 4.57 (brs, 1H), 7.00-7.08 (m, 1H). 13C-NMR (75 MHz, CDCl3): δ (ppm) 23.3, 26.4, 28.4, 28.5, 29.8, 40.4, 45.8, 51.2, 54.8, 70.7, 79.1, 128.3, 129.0, 146.6, 156.0, 162.4. HRMS (ESI): m/z [M+H]+ calcd. for [C16H33N3O3S]+ 383.2111, found: 383.2103. C16H30N4O3S (382.52).

5-((4-(1-Methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)pentan-1-amine (50)

Compound 49 (50 mg, 0.13 mmol) was dissolved in CH2Cl2 (4 mL) and TFA (1 mL) was added. The mixture was stirred at room temperature overnight and cooled to 0 °C followed by the addition of 25%aq NH3 to adjust the pH to 10. The product was extracted with CH2Cl2 (5 × 5 mL). The combined organic phases were dried over Na2SO4 and concentrated under reduced pressure to afford compound 50 as colorless oil (20 mg, 56%), which was used without further purification. \( R_f = 0.3 \) (CH2Cl2/MeOH/25%aq NH3 90:9:1 v/v/v). 1H-NMR (300 MHz, CDCl3): δ (ppm) 1.34-1.55 (m, 4H), 1.73-1.87 (m, 4H), 2.32-2.43 (m, 5H), 2.50 (t, J 5.5 Hz, 2H), 2.66 (t, J 6.7 Hz, 2H), 3.36-3.38 (m, 2H), 4.38 (t, J 6.6 Hz, 2H), 6.92-7.05 (m, 1H). 13C-NMR (75 MHz,
4-Amino-N-(5-((4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)pentyl)butanamide (52)

4-aminobutanoic acid (200 mg, 1.93 mmol) was dissolved in H$_2$O/THF (1:1 v/v) (10 mL) and di-tert-butyl dicarbonate (507 mg, 2.32 mmol) was slowly added followed by the addition of triethylamine (810 µL, 5.82 mmol). The mixture was stirred at room temperature overnight. THF was removed under reduced pressure and 0.1 M aq KHSO$_4$ solution was slowly added to adjust the pH to 3. The product was extracted with ethyl acetate (3 × 10 mL), the combined extracts were dried over Na$_2$SO$_4$ and concentrated under reduced pressure to yield the intermediate 4-((tert-butoxycarbonyl)amino)butanoic acid (compound 51) as colorless oil (270 mg, 69%), which was used without further purification. R$_f$ = 0.8 (CH$_2$Cl$_2$/MeOH/acetic acid 90:9:1 v/v/v). $^1$H-NMR (300 MHz, CDCl$_3$): δ (ppm) 1.43 (s, 9H), 1.75-1.86 (m, 2H), 2.38 (t, J 7.2 Hz, 2H), 3.16 (t, J 6.7 Hz, 2H), 4.75 (brs, 1H), 10.06 (brs, 1H). $^{13}$C-NMR (75 MHz, CDCl$_3$): δ (ppm) 25.1, 28.4, 31.3, 39.8, 60.5, 171.4, 178.4. HRMS (ESI): m/z [M+H]$^+$ calcd. for [C$_{38}$H$_{46}$NO$_3$]$: 522.3147$, found: 522.3147.

51 mg of compound 51 was dissolved in DMF (1 mL) in the presence of dipea (86 µL, 0.49 mmol) and DMF (1 mL) was added HOBt (34 mg, 0.25 mmol), TBTU (80 mg, 0.25 mmol) and diPEA (86 µL, 0.49 mmol) and the mixture was stirred at room temperature for 30 min. Compound 50 (70 mg, 0.25 mmol) dissolved in DMF (1 mL) was added and the mixture was stirred at 60 °C for 3 h. H$_2$O (5 mL) was added, followed by extraction with ethyl acetate (3 × 5 mL). The combined extracts were dried over Na$_2$SO$_4$ and concentrated under reduced pressure to yield the crude product, which was subjected to flash column chromatography (eluent: CH$_2$Cl$_2$/MeOH/25% aq NH$_3$ 90:3:1 v/v/v) to yield the intermediate tert-butyl 4-((4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)pentyl)(amino)-4-oxobutyl)carbamate as yellow oil (80 mg, 67%). R$_f$ = 0.5 (CH$_2$Cl$_2$/MeOH/25% aq NH$_3$ 90:9:1 v/v/v). $^1$H-NMR (300 MHz, CDCl$_3$): δ (ppm) 1.42 (s, 9H), 1.46-1.57 (m, 4H), 1.73-1.80 (m, 2H), 1.82-1.91 (m, 2H), 2.19 (t, J 6.9 Hz, 2H), 2.49-2.55 (m, 5H), 2.63-2.81 (m, 2H), 3.12-3.18 (m, 2H), 3.23-3.29 (m, 2H), 3.51-3.73 (m, 2H), 4.45 (t, J 6.4 Hz, 2H), 4.90 (brs, 1H), 6.37 (brs, 1H), 7.07-7.09 (m, 1H). $^{13}$C-NMR (75 MHz, CDCl$_3$): δ (ppm) 23.4, 26.4, 26.6, 28.4, 28.5, 29.2, 33.6, 39.3, 39.6, 45.8, 51.1, 54.8, 70.7, 79.3, 128.3, 129.0, 146.6, 156.7, 162.4, 172.7. HRMS (ESI): m/z [M+H]$^+$ calcd. for [C$_{22}$H$_{28}$N$_5$O$_4$]$: 468.2639$, found: 468.2650. This intermediate (80 mg, 0.17 mmol) was dissolved in CH$_2$Cl$_2$/TFA (4:1 v/v) (5 mL) and the mixture was stirred at room temperature overnight. CH$_2$Cl$_2$ (5 mL) was added followed by the addition of 25% aq NH$_3$ to adjust the pH of the aqueous phase to 11. The product was extracted with CH$_2$Cl$_2$ (5 × 10 mL). The combined organic phases were dried over Na$_2$SO$_4$ and concentrated under reduced
pressure to give compound 52 as yellow oil (55 mg, 89%), which was used without further purification. Rr = 0.4 (CH₂Cl₂/MeOH/25% aq NH₃ 80:16:1 v/v/v). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 1.38-1.64 (m, 4H), 1.70-1.92 (m, 4H), 1.96 (brs, 2H), 2.22-2.28 (m, 2H), 2.35-2.48 (m, 5H), 2.52-2.56 (m, 2H), 3.00-3.37 (m, 4H), 3.39-3.42 (m, 2H), 4.41 (t, J 6.5 Hz, 2H), 6.88-7.13 (m, 1H), 8.56 (brs, 1H). ¹³C-NMR (75 MHz, CDCl₃): δ (ppm) 23.5, 26.6, 28.5, 29.3, 31.0, 34.1, 39.3, 41.1, 45.9, 51.2, 55.0, 70.6, 128.4, 129.3, 146.8, 162.4, 172.8. HRMS (ESI): m/z[M+H]⁺ calcd. for [C₁₇H₃₀N₅O₂S]⁺ 368.2115, found: 368.2116. C₁₇H₂₉N₅O₂S (367.51)

**N¹,N⁶-Bis(5-((4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-2,5-thiadiazol-3-yl)oxy)pentyl)octanediame (53)**

To a cooled (0 °C) solution of compound 50 (300 mg, 1.06 mmol) and triethylamine (322 mg, 3.18 mmol) in abs. THF (2 mL) was added dropwise octanedioyl dichloride (76 μL, 0.43 mmol) dissolved in abs. THF (1 mL) under an atmosphere of argon. The mixture was stirred at room temperature overnight. The solvent was evaporated. The residue was dissolved in ethyl acetate (5 mL) followed by washing with water. The aqueous phase was treated with ethyl acetate (3 × 10 mL) and the organic extracts were collected. All organic phases were combined and dried over Na₂SO₄. Removal of the solvent under reduced pressure gave the crude product, which was subjected to flash column chromatography (eluent: CH₂Cl₂/MeOH/25% aq NH₃ 97:3:1 v/v/v) to afford compound 53 as white solid (118 mg, 39%), m.p. 55-57 °C. Rr = 0.6 (CH₂Cl₂/MeOH/25% aq NH₃ 90:9:1 v/v/v). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 1.26-1.37 (m, 4H), 1.48-1.65 (m, 10H), 1.78-1.96 (m, 6H), 2.14 (t, J 7.5 Hz, 4H), 2.40-2.50 (m, 10H), 2.57-2.61 (m, 4H), 3.23-3.29 (m, 4H), 3.42-3.49 (m, 4H), 4.44 (t, J 6.5 Hz, 4H), 5.58 (brs, 2H), 7.02-7.05 (m, 2H). ¹³C-NMR (75 MHz, CDCl₃): δ (ppm) 23.4, 25.8, 26.6, 28.5, 28.7, 29.4, 36.6, 39.3, 45.9, 51.2, 55.0, 70.6, 128.4, 129.3, 146.8, 162.4, 173.0. RP-HPLC (220 nm): 96% (tᵣ = 18.1 min, k = 5.3). HRMS (ESI): m/z [M+H]⁺ calcd. for [C₃₄H₅₅N₈O₄S₂]⁺ 703.3782, found: 703.3786. C₃₄H₅₄N₈O₄S₂ (702.98)

**N¹,N₁⁰-Bis(5-((4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)pentyl)decanediamide (54)**

To a cooled (0 °C) solution of compound 50 (400 mg, 1.42 mmol) and triethylamine (430 mg, 4.25 mmol) in abs. THF (5 mL) was added dropwise decanedioyl dichloride (92 μL, 0.57 mmol) dissolved in abs. THF (1 mL) under an atmosphere of argon. The mixture was stirred at room temperature overnight. The solvent was evaporated. The residue was dissolved in ethyl acetate (5 mL) followed by washing with brine. The aqueous phase was treated with ethyl acetate (3 × 10 mL) and the organic extracts were collected. All organic phases were combined and dried over Na₂SO₄. Removal of the solvent under reduced pressure gave the crude product, which was subjected to flash column chromatography (eluent: CH₂Cl₂/MeOH/25% aq
NH₃ 97:3:1 v/v/v) to afford compound 54 as white solid (270 mg, 65%), m.p. 45-49 °C. Rᵣ = 0.5 (CH₂Cl₂/MeOH/25% aq NH₃ 90:9:1 v/v/v). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 1.25-1.33 (m, 8H), 1.41-1.63 (m, 10H), 1.81-1.94 (m, 4H), 2.06-2.23 (m, 4H), 2.12-2.17 (m, 4H), 2.46-2.53 (m, 8H), 2.63-2.67 (m, 4H), 3.27 (dd, J 13, 6.8 Hz, 4H), 3.49-3.54 (m, 4H), 4.44 (t, J 6.5 Hz, 4H), 5.54 (brs, 2H), 7.03-7.07 (m, 2H). ¹³C-NMR (75 MHz, CDCl₃): δ (ppm) 23.4, 25.7, 26.6, 28.5, 29.1, 29.2, 29.4, 36.8, 39.3, 45.9, 51.2, 55.0, 70.6, 128.4, 129.3, 146.8, 162.4, 173.1. RP-HPLC (220 nm): 98% (tᵣ = 19.5 min, k = 5.8). HRMS (ESI): m/z [M+H]⁺ calcd. for [C₃₆H₅₅N₅O₄S₂]+ 731.4097, found: 731.4095. C₃₆H₅₅N₅O₄S₂ (731.0320).

**N⁴,N⁴-Bis(5-((4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)pentyl)terephthalamide (55)**

To a solution of terephthalic acid (117 mg, 0.71 mmol) in DMF (3 mL) were added EDC (271 mg, 1.41 mmol), HOBT (216 mg, 1.41 mmol) and DIPEA (183 mg, 1.42 mmol) and the mixture was stirred at room temperature for 30 min. Compound 50 (400 mg, 1.42 mmol) in DMF (2 mL) was added and stirring was continued at room temperature overnight. H₂O (10 mL) was added followed by extraction with ethyl acetate (3 × 10 mL). The combined extracts were washed with brine, dried over Na₂SO₄ and concentrated under reduced pressure to give the crude product, which was subjected to flash column chromatography (eluent: CH₂Cl₂/MeOH/25% aq NH₃ 95:5:1 v/v/v) to afford compound 55 as white solid (130 mg, 26%), m.p. 50-53 °C. Rᵣ = 0.5 (CH₂Cl₂/MeOH/25% aq NH₃ 95:5:1 v/v/v). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 1.52-1.61 (m, 4H), 1.67-1.78 (m, 4H), 1.83-1.96 (m, 4H), 2.42-2.52 (m, 4H), 2.62 (s, 6H), 2.78 (t, J 5.8 Hz, 4H), 3.46-3.54 (m, 4H), 3.65-3.70 (m, 4H), 4.47 (t, J 6.2 Hz, 4H), 6.94 (brs, 2H), 7.01-7.09 (m, 2H), 7.85 (s, 4H). ¹³C-NMR (75 MHz, CDCl₃): δ (ppm) 23.6, 25.0, 28.5, 29.3, 39.9, 44.9, 50.7, 53.8, 70.9, 127.0, 127.3, 127.8, 137.2, 145.7, 162.4, 166.9. RP-HPLC (220 nm): 96% (tᵣ = 18.3 min, k = 5.4). HRMS (ESI): m/z [M+H]⁺ calcd. for [C₃₆H₄₇N₅O₄S₂]+ 695.3156, found: 695.3158. C₃₆H₄₇N₅O₄S₂ (694.91).

**Ethyl 4-((2-nitrophenyl)amino)piperidine-1-carboxylate (58)**

Ethyl 4-aminopiperidine-1-carboxylate (compound 57) (244 mg, 1.42 mmol) and potassium carbonate (587 mg, 4.25 mmol) were added to a stirred solution of 1-fluoro-2-nitrobenzene (compound 56) (200 mg, 1.42 mmol) in DMF (1.5 mL) followed by the addition of sodium iodide (106 mg, 0.71 mmol). The mixture was stirred at 180 °C under microwave irradiation for 10 min, cooled to room temperature and diluted with water (50 mL). The product was extracted with ethyl acetate (3 × 10 mL). The combined extracts were washed with brine and dried over Na₂SO₄. Removal of the volatiles under reduced pressure gave compound 58 as yellow solid (300 mg, 72%), which was used without further purification. Rᵣ = 0.2 (light petroleum/ethyl acetate 5:1 v/v), m.p. 80-82 °C. ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 1.28 (t, J 7.1 Hz, 3H),
1.51-1.63 (m, 2H), 1.70 (brs, 1H), 2.05-2.11 (m, 2H), 3.04-3.20 (m, 2H), 3.66-3.74 (m, 1H), 4.05-4.19 (m, 4H), 6.63-6.68 (m, 1H), 6.87 (d, J = 8.4 Hz, 1H), 7.37-7.50 (m, 1H), 8.19 (dd, J = 8.6, 1.6 Hz, 1H). 13C-NMR (75 MHz, CDCl3): δ (ppm) 14.7, 31.7, 42.2, 49.1, 61.6, 113.9, 115.5, 127.3, 132.0, 136.3, 144.3, 155.5. HRMS (ESI): m/z [M+H]+ calcd. for \([C_{14}H_{20}N_3O_4]^+\) 294.1448, found: 294.1453. C_{14}H_{19}N_3O_4 (293.32).

**Ethyl 4-((2-aminophenyl)amino)piperidine-1-carboxylate (59)**

A mixture of compound 58 (200 mg, 0.68 mmol), 10% Pd/C (20 mg) and MeOH (10 mL) was stirred in an autoclave (1 L) under an atmosphere of hydrogen at 10 atm at room temperature overnight. The catalyst was removed by filtration through a pad of celite, which was washed with MeOH (2 × 5 mL). The combined filtrates were concentrated under reduced pressure to give compound 59 as purple solid (160 mg, 89%), which was used without further purification. R_f = 0.4 (light petroleum/acetone = 4:1), m.p. 138-140 °C. 1H-NMR (300 MHz, CDCl3): δ (ppm) 1.27 (t, J = 7.1 Hz, 3H), 1.35-1.48 (m, 2H), 1.98-2.12 (m, 2H), 2.90-3.09 (m, 3H), 3.20 (brs, 1H), 3.34-3.51 (m, 2H), 4.07 (brs, 2H), 4.15 (q, J = 12 Hz, 2H). 13C-NMR (75 MHz, CDCl3): δ (ppm) 14.7, 32.3, 42.6, 50.3, 61.4, 113.9, 117.1, 119.6, 120.6, 135.2, 135.3, 155.6. HRMS (ESI): m/z [M+H]+ calcd. for \([C_{14}H_{22}N_3O_2]^+\) 264.1707, found: 264.1718. C_{14}H_{21}N_3O_2 (263.34).

**Ethyl 4-(2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)piperidine-1-carboxylate (60)**

A solution of triphosgene (85 mg, 0.28 mmol) in anhydrous CH2Cl2 (5 mL) was added dropwise over 10 min to a stirred and cooled (0 °C) mixture of compound 59 (50 mg, 0.19 mmol), sodium bicarbonate (24 mg, 0.28 mmol) in CH2Cl2 (10 mL). The mixture was slowly warmed up to room temperature and stirred for additional 2 h. Water (5 mL) was added slowly and the organic phase was separated followed by additional extraction with CH2Cl2 (2 x 10 mL). The combined organic phases were washed with brine, dried over Na2SO4 and evaporated under reduced pressure to give the crude product, which was subjected to column chromatography (eluent: light petroleum/acetone 2:1 v/v) to give compound 60 as white solid (40 mg, 73%). R_f = 0.3 (light petroleum/ethyl acetate 1:1 v/v), m.p. 173-176 °C. 1H-NMR (300 MHz, CDCl3): δ (ppm) 1.30 (t, J = 7.1 Hz, 3H), 1.84-1.95 (m, 2H), 2.30-2.41 (m, 2H), 2.89-2.98 (m, 2H), 4.19 (q, J = 7.1 Hz, 2H), 4.37-4.41 (m, 2H), 4.46-4.57 (m, 1H), 7.05-7.16 (m, 4H), 10.25 (brs, 1H). 13C-NMR (75 MHz, CDCl3): δ (ppm) 14.7, 30.9, 43.6, 50.8, 61.6, 109.4, 110.0, 121.2, 121.5, 128.1, 128.9, 155.5, 206.9. HRMS (ESI): m/z [M+H]+ calcd. for \([C_{15}H_{22}N_3O_3]^+\) 290.1499, found: 290.1515. C_{15}H_{19}N_3O_3 (289.34).

**1-(Piperidin-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one (61)**

Compound 60 (200 mg, 0.69 mmol) was suspended in 10% aq NaOH (16 mL), the mixture
was kept under reflux for 5 h, and cooled to room temperature and acidified by the addition of 10% HCl solution until the evolution of gas had ceased (pH around 2). Afterwards, the pH was carefully adjusted to 9 using 15% NaOH solution, followed by extraction with CH$_2$Cl$_2$ (4 x 10 mL). The combined extracts were dried over Na$_2$SO$_4$ and the solvent was evaporated under reduced pressure to give compound 61 as white solid (120 mg, 81%), m.p. 112-115 °C, which was used without further purification. $R_t = 0.4$ (CH$_2$Cl$_2$/MeOH/25% aq NH$_3$ 90:9:1 v/v/v). $^1$H-NMR (300 MHz, [D$_6$]DMSO): $\delta$ (ppm) 1.56-1.60 (m, 2H), 2.08-2.30 (m, 2H), 2.53-2.61 (m, 2H), 3.04-3.08 (m, 2H), 3.29 (brs, 1H), 4.17-4.28 (m, 1H), 6.92-7.03 (m, 3H), 7.28 (dd, J 7.1, 2.4 Hz, 1H), 10.83 (brs, 1H). $^{13}$C-NMR (75 MHz, [D$_6$]DMSO): $\delta$ (ppm) 29.8, 45.6, 50.1, 108.7, 108.8, 120.1, 120.3, 128.2, 129.0, 153.5. HRMS (ESI): $m/z$ [M+H]$^+$ calcd. for [C$_{12}$H$_{16}$N$_3$O]$^+$ 218.1288, found: 218.1289. C$_{12}$H$_{15}$N$_3$O (217.27).

1-[(1,4'-Bipiperidin)-4-yl]-1,3-dihydro-2H-benzo[d]imidazol-2-one (63)

4-piperidine hydrochloride (5.0 g, 32.55 mmol) and sodium bicarbonate (5.5 g, 65.49 mmol) were added to THF/H$_2$O (1:1 v/v) (150 mL) followed by the slow addition of di-tert-butyl dicarbonate (5.7 g, 26.12 mmol) in THF (20 mL). The mixture was stirred at room temperature overnight. THF was evaporated and the product was extracted with CH$_2$Cl$_2$ (3 x 50 mL). The combined extracts were washed with brine and dried over Na$_2$SO$_4$. The solvent was removed under reduced pressure and the product was purified by column chromatography (eluent: light petroleum/ethyl acetate 7:1 v/v) to yield the intermediate tert-butyl 4-oxopiperidine-1-carboxylate (compound 62) as white solid (6.4 g, 98%). $R_t = 0.8$ (CH$_2$Cl$_2$/MeOH 30:1 v/v). $^1$H-NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 1.49 (s, 9H), 2.43 (t, J 6.0 Hz, 4H), 3.73 (t, J 6.0 Hz, 4H). $^{13}$C-NMR (75 MHz, CDCl$_3$): $\delta$ (ppm) 28.4, 41.2, 43.1, 80.5, 154.6, 208.1. HRMS (ESI): $m/z$ [M+H]$^+$ calcd. for [C$_{10}$H$_{18}$NO$_3$]$^+$ 200.1281, found: 200.1279. The intermediate 62 (1.6 g, 8.03 mmol) and acetic acid (0.16 mL, 2.74 mmol) were added to a stirred and cooled (0 °C) solution of compound 61 (1.2 g, 5.53 mmol) in MeOH (50 mL) and the mixture was stirred at 0 °C for 15 min. Sodium cyanoborohydride (688 mg, 10.95 mmol) was added and the stirred mixture was allowed to warm up to room temperature, followed by stirring overnight. 5% aq KHCO$_3$ (16 mL) was added prior to extraction with CH$_2$Cl$_2$ (3 x 20 mL). The combined organic phases were washed with brine and dried over Na$_2$SO$_4$. The volatiles were removed under reduced pressure and the product was purified by flash column chromatography (eluent: CH$_2$Cl$_2$/MeOH/25% aq NH$_3$ 97:2:1 to 95:4:1 v/v/v) to yield the intermediate tert-butyl 4-(2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)-1,4'-bipiperidinyl-1'-carboxylate (1.15 g, 52%) as white solid. $R_t = 0.7$ (CH$_2$Cl$_2$/MeOH/25% aq NH$_3$ 90:9:1 v/v/v). The Boc-protected intermediate (1.1 g, 2.75 mmol) was dissolved in TFA/CH$_2$Cl$_2$ (1:4 v/v) (15 mL) and the mixture was stirred at room temperature overnight. The pH was carefully adjusted to 11 by adding 25% aq NH$_3$. The two phases were separated and the aqueous phase was treated with CH$_2$Cl$_2$ (5 x 20 mL).
The combined organic phases were dried over Na₂SO₄ and the solvent was removed under reduced pressure. The product was purified by flash column chromatography with (eluent: CH₂Cl₂/MeOH/25% aq NH₃ 90:9:1 v/v/v) to afford compound 63 as white solid (620 mg, 75%). m.p. 180-182 °C. Rf = 0.4 (CH₂Cl₂/MeOH/25% aq NH₃ 90:9:1 v/v/v). ¹H-NMR (300 MHz, [D₄]MeOH): δ (ppm) 1.63-1.88 (m, 4H), 2.05-2.09 (m, 2H), 2.36-2.58 (m, 4H), 2.65-2.74 (m, 1H), 2.84-3.02 (m, 2H), 3.12-3.15 (m, 2H), 3.34-3.42 (m, 2H), 4.24-4.33 (m, 1H), 6.95-7.17 (m, 3H), 7.33-7.39 (m, 1H). ¹³C-NMR (75 MHz, [D₄]MeOH): δ (ppm) 27.1, 29.9, 45.1, 49.9, 52.0, 60.7, 110.5, 110.6, 122.2, 122.5, 129.6, 130.3, 156.2. HRMS (ESI): m/z [M+H]⁺ calcd. for [C₁₇H₂₈N₄O]⁺ 301.2023, found: 301.2025. C₁₇H₂₈N₄O (300.41).

1-(1'- (2-Aminoethyl)-[1,4'-bipiperidin]-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one (64)

Compound 63 (570 mg, 1.89 mmol), tert-butyl (2-bromoethyl) carbamate (compound 30) (508 mg, 2.27 mmol) and potassium carbonate (525 mg, 3.80 mmol) were added to MeCN (60 mL) and the mixture was stirred under reflux overnight. Insoluble material was removed by filtration. The filtrate was concentrated under reduced pressure to yield a yellow oily residue, which was dissolved in CH₂Cl₂ (10 mL) followed by washing with water. The aqueous phase was treated with CH₂Cl₂ (3 × 10 mL) and the organic extracts were collected. All organic phases were combined and dried over Na₂SO₄. Removal of the solvent under reduced pressure yielded a yellow oil, which was subjected to flash column chromatography (eluent: CH₂Cl₂/MeOH/25% aq NH₃ 90:3:1 to 90:9:1 v/v/v) to afford the Boc-protected intermediate as colorless oil (350 mg, 42%). Rf = 0.6 (CH₂Cl₂/MeOH/25% aq NH₃ 90:9:1 v/v/v). ¹H-NMR (300 MHz, [D₄]MeOH): δ (ppm) 1.43 (s, 9H), 1.54-1.67 (m, 2H), 1.72-1.84 (m, 2H), 1.87-1.91 (m, 2H), 2.01-2.10 (m, 2H), 2.27-2.60 (m, 7H), 3.01-3.05 (m, 2H), 3.08-3.24 (m, 4H), 4.20-4.43 (m, 1H), 6.89-7.16 (m, 3H), 7.24-7.57 (m, 1H). ¹³C-NMR (75 MHz, [D₄]MeOH): δ (ppm) 28.8, 28.9, 29.9, 38.7, 50.1, 52.0, 54.4, 58.7, 63.1, 80.2, 110.7, 110.9, 122.3, 122.6, 129.7, 130.3, 156.3, 158.4. HRMS (ESI): m/z [M+H]⁺ calcd. for [C₂₄H₃₆N₂O₄]⁺ 444.2969, found: 444.2966. The intermediate (150 mg, 0.34 mmol) was dissolved in CH₂Cl₂/TFA (4:1 v/v) (5 mL) and the mixture was stirred at room temperature overnight. 25% aq NH₃ was added to adjust the pH to 11 followed by extraction with CH₂Cl₂/MeOH (9:1 v/v) (5 × 10 mL). Removal of the volatiles from the combined extracts in vacuo gave compound 64 as colorless oil (100 mg, 86%), which was used without further purification. Rf = 0.1 (CH₂Cl₂/MeOH/25% aq NH₃ 90:9:1 v/v/v). ¹H-NMR (300 MHz, [D₄]MeOH): δ (ppm) 1.50-1.71 (m, 2H), 1.76-1.79 (m, 2H), 1.89-1.94 (m, 2H), 2.00-2.11 (m, 2H), 2.32-2.52 (m, 7H), 2.74-2.78 (m, 1H), 3.00-3.17 (m, 4H), 3.39-3.45 (m, 1H), 4.18-4.44 (m, 1H), 6.95-7.16 (m, 3H), 7.34-7.52 (m, 1H). ¹³C-NMR (75 MHz, [D₄]MeOH): δ (ppm) 28.9, 29.8, 39.3, 50.2, 52.1, 54.5, 61.2, 63.2, 110.6, 110.9, 122.3, 122.6, 129.7, 130.3, 156.3. HRMS (ESI): m/z [M+H]⁺ calcd. for [C₁₉H₂₉N₃O]⁺ 344.2445, found: 344.2443. C₁₉H₂₉N₃O (343.48).
1-(1′-(2-Bromoacetyl)-[1,4′-bipiperidin]-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one (66)

To a solution of compound 63 (630 mg, 2.09 mmol) in CHCl₃ (50 mL) was added pyridine (762 μL, 9.45 mmol) and the mixture was cooled in an ice bath. 2-Bromoacetyl bromide (compound 65) (820 μL, 9.45 mmol) was added dropwise and stirring was continued at room temperature overnight. H₂O (10 mL) was added and the phases were separated. The organic phase was washed with brine, dried over Na₂SO₄ and the solvent was evaporated to obtain the crude product, which was subjected to flash column chromatography (eluent: CH₂Cl₂/MeOH/25%aq NH₃ 90:3:1v/v/v) to yield compound 66 as colorless oil (800 mg, 91%). Rᵣ = 0.7 (CH₂Cl₂/MeOH/25%aq NH₃ 95:5:1v/v/v). ¹H-NMR (300 MHz, [D₄]MeOH): δ (ppm) 1.35-1.73 (m, 2H), 1.78-1.92 (m, 2H), 2.01 (t, J 11 Hz, 2H), 2.36-2.61 (m, 4H), 2.72 (t, J 13 Hz, 2H), 3.14-3.22 (m, 3H), 3.99-4.10 (m, 3H), 4.26-4.33 (m, 1H), 4.55-4.57 (m, 1H), 7.02-7.09 (m, 3H), 7.33-7.48 (m, 1H). ¹³C-NMR (75 MHz, [D₄]MeOH): δ (ppm) 28.8, 29.5, 29.9, 42.9, 47.4, 52.0, 62.7, 110.6, 110.8, 122.2, 122.6, 129.7, 130.4, 156.3, 167.7. HRMS (ESI): m/z [M+H]⁺ calcd. for [C₁₉H₂₈BrN₂O₃]⁺ 421.1234, found: 421.1244. C₁₉H₂₈BrN₂O₃ (421.34).

1-(1′-(2-(6-Amino-4-methyl-1,4-diazaepan-1-yl)acetyl)-[1,4′-bipiperidin]-4-yl)-1,3-dihydro-2H-benzo[d]imidazol-2-one tetrakis(hydrotrifluoroacetate) (67)

Potassium carbonate (53 mg, 0.38 mmol) was added to a suspension of compound 66 (80 mg, 0.19 mmol) and compound 28 (48 mg, 0.21 mmol) in MeCN (2 mL). The mixture was stirred at 110 °C under microwave irradiation for 30 min and cooled to room temperature. Insoluble material was separated by filtration and washed with CH₂Cl₂ (2 × 10 mL). The combined filtrate and washings were concentrated under reduced pressure yielding a yellow residue, which was dissolved in CH₂Cl₂ (5 mL) followed by washing with water. The aqueous phase was treated with CH₂Cl₂ (3 × 10 mL) and the organic extracts were collected. All organic phases were combined and dried over Na₂SO₄. Removal of the volatiles under reduced pressure gave the Boc-protected intermediate (50 mg, 46%), which was dissolved in CH₂Cl₂/TFA (4:1 v/v) (5 mL). The mixture was stirred at room temperature for 8 h. CH₂Cl₂ (10 mL) was added and the volatiles were evaporated. Purification by preparative HPLC (column: Kinetex XB-C18 5 μm 250 × 21 mm; gradient: 0-30 min: MeCN/0.1%aq TFA 5:95-62:38, tᵣ = 12 min) afforded compound 67 as white fluffy solid (35 mg, 43%). ¹H-NMR (600 MHz, [D₄]MeOH): δ (ppm) 1.63-1.90 (m, 2H), 2.09-2.11 (m, 2H), 2.21-2.22 (m, 2H), 2.68-2.72 (m, 1H), 2.82-2.91 (m, 2H), 3.02 (s, 3H), 3.05-3.21 (m, 2H), 3.25-3.29 (m, 1H), 3.32-3.37 (m, 2H), 3.47-3.58 (m, 3H), 3.59-3.66 (m, 2H), 3.65-3.74 (m, 4H), 3.77-3.82 (m, 1H), 3.96-3.97 (m, 1H), 4.57-4.63 (m, 1H), 4.72-4.75 (m, 1H), 7.03-7.09 (m, 3H), 7.32 (d, J 3.4 Hz, 1H). ¹³C-NMR (150 MHz, [D₄]MeOH): δ (ppm) 27.3, 27.5, 27.9, 41.6, 43.9, 46.7, 50.2, 50.4, 51.9, 58.5, 58.6, 60.6, 64.7, 110.0, 110.7, 115.1 (TFA), 117.0 (TFA), 118.9 (TFA), 120.9 (TFA), 122.4, 122.9, 129.7, 130.1, 156.1, 162.3 (TFA), 162.6 (TFA), 162.8 (TFA), 163.0 (TFA), 170.8. RP-HPLC (220 nm):
98% \( (t_{R} = 11.6 \text{ min}, k = 3.0) \). HRMS (ESI): \( m/z [M+H]^+ \) calcd. for [C\(_{25}\)H\(_{40}\)N\(_2\)O\(_2\)]\(^+\) 470.3238, found: 470.3241. C\(_{25}\)H\(_{39}\)N\(_2\)O\(_2\) · C\(_8\)H\(_4\)F\(_{12}\)O\(_8\) (469.63 + 456.09).

**tert-Butyl 4-(2,2-diphenylacetoxy)piperidine-1-carboxylate (70)**

Di-tert-butyl dicarbonate (5.6 g, 25.68 mmol) in THF (20 mL) was slowly added to a solution of piperidin-4-ol (2.0 g, 19.77 mmol) and triethylamine (3.6 mL, 25.70 mmol) in THF/H\(_2\)O (1:7 v/v) (200 mL) and the mixture was stirred at room temperature overnight. THF was removed by evaporation followed by extraction with CH\(_2\)Cl\(_2\) (3 × 50 mL). The combined extracts were dried over Na\(_2\)SO\(_4\) and concentrated under reduced pressure. The residue was subjected to column chromatography (eluent: CH\(_2\)Cl\(_2\)/MeOH 30:1 to 15:1 v/v) to yield the Boc-protected intermediate (compound 69) as white solid (3.7 g, 93%). Compound 69 (2.9 g, 14.41 mmol) and 2,2-diphenylacetic acid (compound 68) (2.7 g, 12.74 mmol) were dissolved in CH\(_2\)Cl\(_2\) (100 mL) and the solution was cooled to 0 \(^\circ\)C. DMAP (173 mg, 1.42 mmol) was added and the mixture was allowed to clear up before the slow addition of \( N,N\)-dicyclohexylcarbodiimide (3.2 g, 15.51 mmol) under stirring at 0 \(^\circ\)C. The mixture was slowly warmed up to room temperature and kept under stirring overnight. H\(_2\)O (50 mL) was added, the phases were separated and the aqueous phase was treated with CH\(_2\)Cl\(_2\) (3 × 20 mL). The combined organic phases were washed with brine, dried over Na\(_2\)SO\(_4\) and concentrated under reduced pressure to give the crude product, which was subjected to column chromatography (eluent: light petroleum/acetone 3:1 v/v) to afford compound 70 as yellow oil (4.9 g, 97%). \( R_f = 0.8 \) (light petroleum/acetone 3:1 v/v). \(^1\)H-NMR (300 MHz, CDCl\(_3\)): \( \delta \) (ppm) 1.40 (s, 9H), 1.49-1.59 (m, 2H), 1.72-1.82 (m, 2H), 3.15-3.24 (m, 2H), 3.15-3.24 (m, 2H), 4.93-5.00 (m, 2H), 7.18-7.29 (m, 10H). \(^13\)C-NMR (75 MHz, CDCl\(_3\)): \( \delta \) (ppm) 28.4, 30.3, 57.3, 65.9, 70.4, 79.7, 127.3, 128.5, 128.6, 138.6, 154.7, 171.8. HRMS (ESI): \( m/z [M+Na]^+ \) calcd. for [C\(_{24}\)H\(_{29}\)N\(_2\)O\(_4\)]\(^+\) 418.1989, found: 418.1988. C\(_{24}\)H\(_{29}\)NO\(_4\) (395.50).

**Piperidin-4-yl 2, 2-diphenylacetate (71)**

Compound 70 (860 mg, 2.17 mmol) was dissolved in CH\(_2\)Cl\(_2\) (40 mL) and the solution was cooled to 0 \(^\circ\)C. TFA (10 mL) was added dropwise, the mixture was allowed to warm up to room temperature and stirring was continued for 8 h. Ice water (10 mL) was added followed by the slow addition of 25% aq NH\(_3\) to adjust the pH value to 11. The product was extracted with CH\(_2\)Cl\(_2\) (3 × 15 mL), the combined organic phases were dried over Na\(_2\)SO\(_4\) and concentrated under reduced pressure to give compound 71 as white solid (360 mg, 56%), m.p. 75-77 \(^\circ\)C. \( R_f = 0.6 \) (CH\(_2\)Cl\(_2\)/MeOH/25% aq NH\(_3\) 90:9:1 v/v/v). \(^1\)H-NMR (300 MHz, CDCl\(_3\)): \( \delta \) (ppm) 1.45-1.65 (m, 2H), 1.82 (brs, 1H), 1.84-1.93 (m, 2H), 2.64-2.73 (m, 2H), 2.89-3.03 (m, 2H), 4.90-4.99 (m, 1H), 5.01 (s, 1H), 7.21-7.35 (m, 10H). \(^13\)C-NMR (75 MHz, CDCl\(_3\)): \( \delta \) (ppm) 31.9, 43.9, 57.4, 71.4, 127.3, 128.6, 128.7, 138.9, 171.9. HRMS (ESI): \( m/z [M+H]^+ \) calcd. for [C\(_{19}\)H\(_{22}\)NO\(_2\)]\(^+\)
1-(2-((tert-Butoxycarbonyl)amino)ethyl)piperidin-4-yl 2,2-diphenylacetate (72)

Compound 71 (150 mg, 0.51 mmol), tert-butyl (2-bromoethyl) carbamate (compound 30) (136 mg, 0.61 mmol) and potassium carbonate (140 mg, 1.01 mmol) were added to MeCN (50 mL) and the mixture was refluxed for 3 h. Insoluble material was separated by filtration and washed with CH₂Cl₂ (2 × 5 mL). The combined filtrate and washings were concentrated under reduced pressure to yield a brown residue, which was dissolved in CH₂Cl₂ (10 mL) followed by washing with water. The aqueous phase was treated with CH₂Cl₂ (3 × 5 mL) and the organic extracts were collected. All organic phases were combined and dried over Na₂SO₄. Removal of the solvent under reduced pressure gave the crude product, which was subjected to flash column chromatography (eluent: CH₂Cl₂/MeOH/25% aq NH₃ 90:3:1 v/v/v) to yield compound 72 as colorless oil (150 mg, 67%). Rₛ = 0.6 (CH₂Cl₂/MeOH/25% aq NH₃ 90:10:1 v/v/v). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 1.40 (s, 9H), 1.58-1.70 (m, 2H), 1.81-1.95 (m, 2H), 2.21-2.30 (m, 2H), 2.39 (t, J 12 Hz, 2H), 2.49-2.59 (m, 2H), 3.16-3.18 (m, 2H), 4.82-4.89 (m, 1H), 4.96 (s, 1H), 5.03 (brs, 1H), 7.18-7.28 (m, 10H).


1-(2-Aminoethyl)piperidin-4-yl 2,2-diphenylacetate (73)

Compound 72 (500 mg, 1.14 mmol) was dissolved in CH₂Cl₂ (4 mL), TFA (1 mL) was added slowly and the mixture was stirred at room temperature for 8 h. 25% aq NH₃ was added slowly to adjust the pH to 11, followed by extraction with CH₂Cl₂/MeOH (9:1 v/v) (5 × 10 mL). The combined extracts were dried over Na₂SO₄ and the volatiles were evaporated to afford compound 73 as colorless oil (320 mg, 83%), which was used without further purification. Rₛ = 0.3 (CH₂Cl₂/MeOH/25% aq NH₃ 90:10:1 v/v/v). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 1.59-1.77 (m, 2H), 1.84-1.93 (m, 2H), 2.26 (t, J 8.5 Hz, 2H), 2.38-2.47 (m, 2H), 2.55 (brs, 2H), 2.79 (t, J 6.0 Hz, 4H), 4.84-4.92 (m, 1H), 5.00 (s, 1H), 7.23-7.26 (m, 2H), 7.28-7.37 (m, 8H). ¹³C-NMR (75 MHz, CDCl₃): δ (ppm) 30.6, 38.5, 50.5, 57.3, 59.5, 70.7, 127.2, 128.57, 128.62, 138.7, 171.9. HRMS (ESI): m/z [M+H]+ calcd. for [C₂₁H₂₆N₂O₂]+ 339.2067, found: 339.2072. C₂₁H₂₆N₂O₂ (338.45).

2-Bromoethyl 9H-xanthene-9-carboxylate (75)

9H-xanthene-9-carboxylic acid (compound 74) (1.0 g, 4.42 mmol) and 2-bromoethan-1-ol (1.1 g, 8.87 mmol) were dissolved in CH₂Cl₂ (30 mL) and the solution was cooled to 0 °C. N,N'-Dicyclohexylcarbodiimide (1.1 g, 5.34 mmol) dissolved in CH₂Cl₂ (5 mL) was added dropwise followed by the addition of DMAP (270 mg, 2.21 mmol). The mixture was allowed to warm up
to room temperature and stirring was continued overnight. H₂O (50 mL) was added, the phases were separated and the aqueous phase was treated with CH₂Cl₂ (3 × 30 mL). The combined organic phases were washed with brine, dried over Na₂SO₄ and concentrated under reduced pressure. Purification by column chromatography (eluent: light petroleum/acetone 7:1 v/v) afforded compound 75 as colorless oil (1.0 g, 68%). Rₚ = 0.7 (light petroleum/acetone 3:1 v/v). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 3.40 (t, J 6.1 Hz, 2H), 4.35 (t, J 6.1 Hz, 2H), 5.06 (s, 1H), 7.06-7.19 (m, 4H), 7.27-7.36 (m, 4H). ¹³C-NMR (75 MHz, CDCl₃): δ (ppm) 28.3, 45.2, 64.5, 117.1, 117.9, 123.4, 129.1, 129.3, 151.4, 171.4. HRMS (ESI): m/z [M+H]⁺ calcd. for [C₁₆H₁₄BrO₃]⁺ 333.0121, found: 333.0124. C₁₆H₁₃BrO₃ (333.18).

4-Bromobutyl 9H-xanthene-9-carboxylate (76)

9H-Xanthene-9-carboxylic acid (compound 74) (2.0 g, 8.84 mmol) and 4-bromobutan-1-ol (1.6 g, 10.61 mmol) were dissolved in CH₂Cl₂ (30 mL) and the mixture was cooled to 0 °C. A solution of N, N'-dicyclohexylcarbodiimide (2.2 g, 10.61 mmol) in CH₂Cl₂ (5 mL) was added dropwise followed by the addition of DMAP (270 mg, 2.21 mmol). The mixture was allowed to warm up to room temperature and stirring was continued overnight. H₂O (20 mL) was added, the phases were separated and the aqueous phase was treated with CH₂Cl₂ (3 × 15 mL). The combined organic phases were washed with brine, dried over Na₂SO₄ and concentrated under reduced pressure. Purification by column chromatography (eluent: light petroleum/acetone 7:1 v/v) yielded compound 76 as colorless oil (1.8 g, 56%). Rₚ = 0.7 (light petroleum/acetone 4:1 v/v). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 1.59-1.74 (m, 4H), 3.23 (t, J 9.0 Hz, 2H), 4.06 (t, J 6.0 Hz, 2H), 4.99 (s, 1H), 7.04-7.18 (m, 4H), 7.26-7.34 (m, 4H). ¹³C-NMR (75 MHz, CDCl₃): δ (ppm) 27.1, 29.0, 33.0, 45.7, 64.5, 117.1, 118.5, 123.4, 128.9, 129.3, 151.4, 171.9. HRMS (ESI): m/z [M+H]⁺ calcd. for [C₁₈H₁₈BrO₃]⁺ 361.0434, found: 361.0435. C₁₈H₁₇BrO₃ (361.24).

2-(Piperazin-1-yl)ethyl 9H-xanthene-9-carboxylate (77)

Compound 75 (500 mg, 1.50 mmol), piperazine (1.04 g, 12.08 mmol) and potassium carbonate (416 mg, 3.01 mmol) were added to MeCN (18 mL) and the mixture was refluxed overnight. Insoluble material was separated by filtration and washed with CH₂Cl₂ (2 × 10 mL). The combined filtrate and washings were concentrated under reduced pressure yielding a yellow oil, which was dissolved in CH₂Cl₂ (10 mL) followed by washing with brine. The aqueous phase was treated with CH₂Cl₂ (3 × 15 mL) and the organic extracts were collected. All organic phases were combined and dried over Na₂SO₄. Removal of the solvent under reduced pressure gave the crude product, which was subjected to column chromatography (eluent: CH₂Cl₂/MeOH/25%aq NH₃ 96:3:1 v/v/v) to yield compound 77 as yellow solid (300 mg, 59%). Rₚ = 0.5 (CH₂Cl₂/MeOH/25%aq NH₃ 90:9:1 v/v/v), m.p. 77-79 °C. ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 2.36-2.54 (m, 6H), 2.82-2.92 (m, 4H), 4.61 (brs, 1H), 4.13 (t, J 10 Hz, 2H), 5.00 (s,
H), 7.05-7.17 (m, 4H), 7.27-7.35 (m, 4H).$^{13}$C-NMR (75 MHz, CDCl$_3$): $\delta$ (ppm) 44.2, 45.6, 50.9, 56.3, 62.7, 117.0, 118.4, 123.3, 128.9, 129.2, 151.4, 171.5. HRMS (ESI): m/z [M+H]$^+$ calcd. for [C$_{20}$H$_{23}$N$_2$O$_3$]$^+$ 339.1703, found: 339.1707. C$_{20}$H$_{22}$N$_2$O$_3$ (338.41).

4-(Piperazin-1-yl)butyl 9H-xanthene-9-carboxylate (78)

Compound 76 (1.0 g, 2.77 mmol), piperazine (1.9 g, 22.07 mmol) and potassium carbonate (1.2 g, 8.70 mmol) were added to MeCN (50 mL) and the stirred mixture was kept under reflux for 1.5 h. Insoluble material was separated by filtration and washed with CH$_2$Cl$_2$ (2 x 20 mL). The combined filtrate and washings were concentrated under reduced pressure to give a yellow oil, which was dissolved in CH$_2$Cl$_2$ (20 mL) followed by washing with water. The aqueous phase was treated with CH$_2$Cl$_2$ (3 x 30 mL) and the organic extracts were collected. All organic phases were combined and dried over Na$_2$SO$_4$. Removal of the volatiles under reduced pressure gave the crude product, which was subjected to column chromatography (eluent: CH$_2$Cl$_2$/MeOH/25%aq NH$_3$ 94:5:1 v/v/v) to afford compound 78 as colorless oil (470 mg, 46%). $R_f$ = 0.5 (CH$_2$Cl$_2$/MeOH/25%aq NH$_3$ 90:9:1 v/v/v). $^1$H-NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 1.19-1.42 (m, 2H), 1.43-1.60 (m, 2H), 2.12-2.26 (m, 4H), 2.26-2.32 (m, 2H), 2.36 (brs, 1H), 2.81-2.94 (m, 4H), 4.04 (t, $J$ 6.3 Hz, 2H), 4.98 (s, 1H), 7.00-7.17 (m, 4H), 7.23-7.35 (m, 4H). $^{13}$C-NMR (75 MHz, CDCl$_3$): $\delta$ (ppm) 22.6, 26.5, 45.6, 45.8, 54.1, 58.4, 65.3, 116.9, 118.5, 123.3, 128.9, 129.1, 151.3, 171.9. HRMS (ESI): m/z [M+H]$^+$ calcd. for [C$_{22}$H$_{27}$N$_2$O$_3$]$^+$ 367.2016, found: 367.2027. C$_{22}$H$_{26}$N$_2$O$_3$ (366.46).

2-(4-(4-(tert-Butoxycarbonyl)amino)ethyl)piperazin-1-yl)ethyl-9H-xanthene-9-carboxylate (79)

Compound 77 (1.27 g, 3.76 mmol), tert-butyl (2-bromoethyl) carbamate (compound 30) (921 mg, 4.13 mmol) and potassium carbonate (1.3 g, 9.41 mmol) were added to MeCN (30 mL) and the mixture was kept under reflux for 2 h. Insoluble material was separated by filtration and washed with CH$_2$Cl$_2$ (2 x 10 mL). The filtrate and washings were combined and the volatiles were removed under reduced pressure yielding a yellow oily residue, which was dissolved in CH$_2$Cl$_2$ (20 mL) followed by washing with water. The aqueous phase was treated with CH$_2$Cl$_2$ (3 x 30 mL) and the organic extracts were collected. All organic phases were combined and dried over Na$_2$SO$_4$. Removal of the solvent under reduced pressure gave the crude product, which was subjected to column chromatography (eluent: CH$_2$Cl$_2$/MeOH/25%aq NH$_3$ 90:3:1 v/v/v) to afford compound 79 as yellow oil (1.03 g, 57%). $R_f$ = 0.5 (CH$_2$Cl$_2$/MeOH/25%aq NH$_3$ 90:10:1 v/v/v). $^1$H-NMR (300 MHz, CDCl$_3$): $\delta$ (ppm) 1.45 (d, $J$ 5.3 Hz, 9H), 2.13-2.38 (m, 8H), 2.42 (t, $J$ 5.9 Hz, 2H), 2.45-2.55 (m, 2H), 3.19-3.27 (m, 2H), 4.08-4.19 (m, 2H), 4.99 (s, 1H), 5.04 (brs, 1H), 7.04-7.11 (m, 2H), 7.04-7.13 (m, 2H), 7.23-7.31 (m, 4H). $^{13}$C-NMR (75 MHz, CDCl$_3$): $\delta$ (ppm) 28.5, 37.0, 45.5, 52.8, 53.5, 56.3, 57.0, 63.3, 79.2,
2-(4-(2-Aminopropyl)piperazin-1-yl)ethyl 9H-xanthene-9-carboxylate (80)

Compound 79 (1.0 g, 2.08 mmol) was dissolved in CH$_2$Cl$_2$ (8 mL), TFA (2 mL) was added slowly, and the mixture was stirred at room temperature overnight. 25% aq NH$_3$ was added to adjust the pH to 11, followed by extraction with CH$_2$Cl$_2$/MeOH (9:1 v/v) (5 × 15 mL). The combined extracts were dried over Na$_2$SO$_4$. Removal of the solvent in vacuo gave compound 80 as colorless oil (700 mg, 88%), which was used without further purification. R$_f$ = 0.2 (CH$_2$Cl$_2$/MeOH/25% aq NH$_3$ 90:10:1 v/v/v). $^1$H-NMR (300 MHz, [D$_4$]MeOH): δ (ppm) 2.20-2.42 (m, 8H), 2.41-2.61 (m, 4H), 2.67-2.79 (m, 2H), 4.05-4.23 (m, 2H), 4.90 (s, 1H), 7.01-7.21 (m, 4H), 7.23-7.44 (m, 4H). $^{13}$C-NMR (75 MHz, [D$_4$]MeOH): δ (ppm) 38.9, 46.1, 53.9, 54.0, 57.4, 60.9, 64.4, 117.8, 119.9, 124.6, 130.32, 130.34, 152.9, 173.2. HRMS (ESI): m/z[M+H]$^+$ calcd. for [C$_{23}$H$_{28}$N$_3$O$_3$]$^+$ 382.2125, found: 382.2123. C$_{22}$H$_{27}$N$_3$O$_3$ (381.48).

1-(3-Chloropropyl)-3,4-dihydroquinolin-2(1H)-one (83)$^a$

3,4-dihydroquinolin-2(1H)-one (compound 81) (1.0 g, 6.80 mmol), 1-chloro-3-iodopropane (compound 82) (1.7 mg, 8.32 mmol) and caesium carbonate (4.4 g, 13.51 mmol) were added to MeCN (50 mL) and the mixture was stirred and heated to 50 °C for 12 h. Insoluble material was separated by filtration and washed with CH$_2$Cl$_2$ (2 × 20 mL). The filtrate and washings were combined and the volatiles were removed under reduced pressure yielding a yellow solid, which was dissolved in CH$_2$Cl$_2$ (20 mL). This solution was washed with brine, the phases were separated and the aqueous phase was treated with CH$_2$Cl$_2$ (3 × 20 mL). The combined organic phases were dried over Na$_2$SO$_4$ and concentrated under reduced pressure to yield a yellow oil, which was subjected to column chromatography (eluent: CH$_2$Cl$_2$/MeOH/25% aq NH$_3$ 90:3:1 v/v/v) to afford compound 83 as yellow oil (1.05 g, 69%). R$_f$ = 0.7 (CH$_2$Cl$_2$/MeOH/25% aq NH$_3$ 90:3:1). $^1$H-NMR (300 MHz, [D$_4$]MeOH): δ (ppm) 2.07-2.21 (m, 2H), 2.59-2.70 (m, 2H), 2.81-2.98 (m, 2H), 3.56-3.71 (m, 2H), 4.00-4.20 (m, 2H), 6.97-7.10 (m, 2H), 7.15-7.31 (m, 2H). $^{13}$C-NMR (75 MHz, [D$_4$]MeOH): δ (ppm) 25.5, 30.2, 31.8, 40.1, 42.8, 114.6, 122.9, 126.5, 127.6, 128.1, 139.4, 170.4. HRMS (ESI): m/z [M+H]$^+$ calcd. for [C$_{12}$H$_{15}$ClNO]$^+$ 224.0837, found: 224.0846. C$_{12}$H$_{13}$ClNO (223.70).

1-(3-(4-(4-Hydroxybutyl)piperidin-1-yl)propyl)-3,4-dihydroquinolin-2(1H)-one (85)

4-(Piperidin-4-yl)butanoic acid hydrochloride (1.0 g, 4.81 mmol) was suspended in anhydrous THF (20 mL) under an atmosphere of argon. The suspension was immersed in an ice bath and lithium aluminium hydride (456 mg, 12.01 mmol) was added in portions under stirring. The mixture was slowly warmed up to room temperature, then kept under reflux overnight, and
cooled in an ice bath. For quenching, water (5 mL), 15% NaOH solution (10 mL) and water (10 mL) were added dropwise to reaction mixture. Insoluble material was separated by filtration and washed with chloroform (3 × 20 mL). The combined filtrate and washings were dried over Na₂SO₄ and concentrated under reduced pressure to give the intermediate 4-(piperidin-4-yl)butan-1-ol 88 (compound 84) as colorless oil-like residue (510 mg, 68%), which was used without further purification. Rf = 0.1 (CH₂Cl₂/MeOH/25% aq NH₃ 66:33:1 v/v/v). ¹H-NMR (300 MHz, [D₆]MeOH): δ (ppm) 1.04-1.18 (m, 2H), 1.20-1.31 (m, 2H), 1.31-1.44 (m, 3H), 1.47-1.57 (m, 2H), 1.70 (d, J 12 Hz, 2H), 2.55-2.57 (m, 2H), 2.99-3.01 (m, 2H), 3.54 (t, J 6.5 Hz, 2H). ¹³C-NMR (75 MHz, [D₆]MeOH): δ (ppm) 23.9, 33.8, 33.9, 37.2, 38.2, 47.1, 62.9. HRMS (ESI): m/z [M+H]+ calcd. for [C₁₂H₁₇NO]+ 158.1539, found: 158.1541. The intermediate 84 (867 mg, 5.52 mmol) and compound 83 (1.1 g, 4.92 mmol) were dissolved in MeCN (30 mL), followed by the addition of potassium carbonate (1.4 g, 10.14 mmol) and sodium iodide (376 mg, 2.51 mmol). The mixture was kept at 50 °C for 24 h. Insoluble material was separated by filtration and washed with CH₂Cl₂ (2 × 10 mL). The filtrate and washings were combined and the solvent was removed under reduced pressure to yield a yellow residue, which was dissolved in CH₂Cl₂ (20 mL) followed by washing with water. The aqueous phase was treated with CH₂Cl₂ (3 × 20 mL) and the organic extracts were collected. All organic phases were combined and dried over Na₂SO₄. Removal of the solvent under reduced pressure gave crude product, which was subjected to column chromatography (eluent: CH₂Cl₂/MeOH/25% aq NH₃ 90:9:1 v/v/v) to afford compound 85 as colorless oil (900 mg, 53%). Rf = 0.4 (CH₂Cl₂/MeOH/25% aq NH₃ 90:10:1 v/v/v). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 1.26-1.42 (m, 7H), 1.45-1.59 (m, 2H), 1.68 (brs, 1H), 1.68-1.72 (m, 2H), 1.84-2.12 (m, 4H), 2.45-2.56 (m, 2H), 2.59-2.65 (m, 2H), 2.77-2.94 (m, 2H), 2.99-3.03 (m, 2H), 3.62 (t, J 6.4 Hz, 2H), 3.90-4.00 (m, 2H), 6.96-7.01 (m, 1H), 7.07 (d, J 7.7 Hz, 1H), 7.13-7.16 (m, 1H), 7.20-7.26 (m, 1H). ¹³C NMR (75 MHz, CDCl₃): δ (ppm) 18.4, 22.9, 24.4, 25.4, 31.8, 32.9, 36.0, 40.5, 50.8, 53.9, 55.9, 62.8, 114.9, 122.9, 126.4, 127.6, 128.0, 139.4, 170.4. HRMS (ESI): m/z [M+H]+ calcd. for [C₂₁H₃₂N₂O₂]⁺ 345.2537, found: 345.2565. C₂₁H₃₂N₂O₂ (344.50).

1-(3-(4-(4-Bromobutyl)piperidin-1-yl)propyl)-3,4-dihydroquinolin-2(1H)-one (86)

Compound 85 (900 mg, 2.61 mmol) and PPh₃ (2.06 g, 7.86 mmol) were dissolved in anhydrous CH₂Cl₂ (30 mL) and the solution was cooled to -5 °C. A solution of CBr₄ (3.03 g, 9.14 mmol) in anhydrous CH₂Cl₂ (15 mL) was slowly dropped into the stirred mixture, thereby keeping the temperature of the mixture below 5 °C. Stirring was continued at room temperature overnight. The solvent was evaporated yielding a yellow residue, which was subjected to column chromatography (eluent: light petroleum/acetone/25% aq NH₃ 80:20:1 v/v/v) to yield compound 86 as colorless oil (330 mg, 31%). Rf = 0.3 (light petroleum/acetone/25% aq NH₃ 80:20:1 v/v/v). ¹H-NMR (300 MHz, CDCl₃): δ (ppm) 1.26-1.31 (m, 5H), 1.35-1.52 (m, 2H), 1.68
(d, J 9.3 Hz, 2H), 1.76-2.03 (m, 6H), 2.42 (t, J 6.1 Hz, 2H), 2.60-2.65 (m, 2H), 2.80-3.01 (m, 4H), 3.40 (t, J 6.8 Hz, 2H), 3.96 (t, J 7.5Hz, 2H), 6.96-7.01 (m, 1H), 7.08 (d, J 8.1 Hz, 1H), 7.15 (dd, J 7.3, 1.1 Hz, 1H), 7.22 (dd, J 11, 4.6 Hz, 1H). \(^{13}\)C-NMR (75 MHz, CDCl\(_3\)): \(\delta\) (ppm) 24.8, 25.5, 25.7, 32.0, 32.2, 33.0, 34.1, 35.6, 35.7, 40.7, 54.1, 56.1, 115.1, 122.9, 126.6, 127.6, 128.1, 139.7, 170.4. HRMS (ESI): \(m/z\) [M+H]^+ calcd. for [C\(_{21}\)H\(_{31}\)BrN\(_2\)O]^+ 407.1693, found: 407.1695. C\(_{21}\)H\(_{31}\)BrN\(_2\)O (407.40).

tert-Butyl (2-(4-(1-(3-(2-oxo-3,4-dihydroquinolin-1(2H)-yl)propyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)carbamate (87)

Compound 86 (1.21 g, 2.97 mmol), tert-butyl (2-(piperazin-1-yl)ethyl)carbamate (compound 32) (1.5 g, 6.55 mmol) and potassium carbonate (1.24 g, 8.99 mmol) were added to MeCN (60 mL) and the stirred mixture was kept under reflux for 2 h. Insoluble material was removed by filtration and the filtrate was concentrated under reduced pressure yielding a yellow oily residue, which was dissolved in CH\(_2\)Cl\(_2\) (20 mL) followed by washing with water. The aqueous phase was treated with CH\(_2\)Cl\(_2\) (3 × 30 mL) and the organic extracts were collected. All organic phases were combined and dried over Na\(_2\)SO\(_4\). Removal of the solvent under reduced pressure gave the crude product, which was subjected to column chromatography (eluent: CH\(_2\)Cl\(_2\)/MeOH/25% aq NH\(_3\) 90:3:1 v/v/v) to afford compound 87 as yellow oil (1.02 g, 62%). \(R_t = 0.5\) (CH\(_2\)Cl\(_2\)/MeOH/25% aq NH\(_3\) 90:9:1 v/v/v). \(^1\)H-NMR (300 MHz, CDCl\(_3\)): \(\delta\) (ppm) 1.13-1.18 (m, 7H), 1.38 (s, 9H), 1.39-1.44 (m, 2H), 1.53-1.62 (m, 2H), 1.71-1.94 (m, 4H), 2.19-2.29 (m, 2H), 2.30-2.51 (m, 10H), 2.54-2.59 (m, 3H), 2.74-2.94 (m, 4H), 3.08-3.21 (m, 2H), 3.90 (t, J 6.0 Hz, 2H), 6.89-6.95 (m, 1H), 7.02-7.09 (m, 2H), 7.05-7.11 (m, 1H), 7.12-7.20 (m, 1H). \(^{13}\)C-NMR (75 MHz, CDCl\(_3\)): \(\delta\) (ppm) 24.7, 25.6, 27.1, 28.5, 31.9, 32.2, 35.6, 36.4, 37.1, 40.5, 52.9, 53.2, 54.1, 56.1, 57.1, 58.5, 65.9, 79.1, 114.9, 122.7, 126.5, 127.5, 127.9, 139.6, 155.9, 170.2. HRMS (ESI): \(m/z\) [M+H]^+ calcd. for [C\(_{32}\)H\(_{54}\)N\(_3\)O\(_3\)]^+ 556.4221, found: 556.4227. C\(_{32}\)H\(_{53}\)N\(_3\)O\(_3\) (555.81)

1-(3-(4-(4-(2-Aminoethyl)piperazin-1-yl)butyl)piperidin-1-yl)propyl)-3,4-dihydroquinolin-2(1H)-one (88)

Compound 87 (300 mg, 0.54 mmol) was dissolved in CH\(_2\)Cl\(_2\)/TFA (4:1 v/v) (5 mL) and the mixture was stirred at room temperature for 8 h. 25% aq NH\(_3\) was added to adjust the pH to 11, followed by extraction with CH\(_2\)Cl\(_2\)/MeOH (9:1 v/v) (5 × 10 mL). The combined extracts were dried over Na\(_2\)SO\(_4\). Removal of the volatiles in vacuo yielded compound 88 as yellow oil (240 mg, 97%), which was used without further purification. \(R_t = 0.1\) (CH\(_2\)Cl\(_2\)/MeOH/25% aq NH\(_3\) 90:9:1 v/v/v). \(^1\)H-NMR (300 MHz, [D\(_3\)]MeOH): \(\delta\) (ppm) 1.16-1.32 (m, 7H), 1.44-1.54 (m, 2H), 1.62-1.70 (m, 2H), 1.76-1.86 (m, 2H), 1.89-1.98 (m, 2H), 2.32-2.40 (m, 6H), 2.42-2.67 (m, 10H), 2.78-2.82 (m, 2H), 2.81-2.98 (m, 4H), 3.86-4.09 (m, 2H), 6.89-7.08 (m, 1H), 7.08-7.31(m,
3H). $^{13}$C-NMR (75 MHz, [D$_2$]MeOH): δ (ppm) 25.5, 25.8, 26.3, 27.7, 32.8, 33.0, 36.8, 37.6, 38.7, 41.3, 53.9, 55.1, 55.0, 57.1, 59.8, 60.1, 116.5, 124.4, 128.3, 128.7, 129.2, 140.4, 170.5. HRMS (ESI): m/z [M+H]$^+$ calcd. for [C$_{27}$H$_{48}$N$_3$O]$^+$ 456.3697, found: 456.3700. C$_{27}$H$_{48}$N$_3$O (455.69).

5,10-Dihydro-11H-dibenzo[b,e][1,4]diazepin-11-one (91)$^{54}$

A mixture of 2-chlorobenzoic acid (compound 89) (20 g, 127.7 mmol), 1,2-benzenediamine (compound 90) (13.8 g, 127.6 mmol) and copper powder (8.1 g, 127.5 mmol) in chlorobenzene (300 mL) was kept under reflux in a round bottom flask, equipped with a Dean-stark apparatus, for 6 h. The hot mixture was filtered and the solid was washed with a small amount of chlorobenzene. The combined filtrates were concentrated under reduced pressure to reach a volume of approx. 400 mL. After storage at -20 °C for 12 h the crystalline product was collected and recrystallized from ethanol/ethyl acetate (1:1 v/v) to yield 91 as yellow-green crystals (3.5 g, 13%). R$_f$ = 0.6 (light petroleum/ethyl acetate 1:1 v/v), m.p. 247-249 °C (Lit$^{54}$, m.p. 256-257 °C). $^1$H-NMR (300 MHz, [D$_6$]DMSO): (ppm) 6.85-7.02 (m, 6H), 7.26-7.40 (m, 1H), 7.67 (dd, J 7.9, 1.6 Hz, 1H), 7.85 (s, 1H), 9.85 (s, 1H). $^{13}$C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 118.9, 119.7, 120.6, 121.2, 122.6, 122.7, 124.4, 129.7, 131.9, 133.1, 139.9, 150.3, 167.8. HRMS (ESI): m/z [M+H]$^+$ calcd. for [C$_{13}$H$_{11}$N$_2$O]$^+$ 211.0866, found: 211.0862. C$_{13}$H$_{10}$N$_2$O (210.24).

5-(2-Chloroacetyl)-5,10-dihydro-11H-dibenzo[b,e][1,4]diazepin-11-one (92)$^{69}$

Under an atmosphere of argon compound 91 (3.5 g, 16.65 mmol), N,N-dimethylaniline (1.61 g, 13.28 mmol) and 2-chloroacetyl chloride (6.59 g, 58.35 mmol) were added to abs. THF (50 mL) and the mixture was kept under reflux overnight. 5% aq KHCO$_3$ (40 mL) was added slowly, resulting in the formation of a greyish solid, which was collected by filtration. The filtrate was concentrated under reduced pressure yielding a white-pink solid, which was washed with light petroleum/ethyl acetate (1:1 v/v) until the color of the solid turned to grey. The grey solids were combined and dried under vacuum at 40°C for 4 h. The hot mixture was kept under reflux in a round bottom flask, equipped with a Dean-stark apparatus, for 6 h. The hot mixture was filtered and the solid was washed with a small amount of chlorobenzene. The combined filtrates were concentrated under reduced pressure to reach a volume of approx. 400 mL. After storage at -20 °C for 12 h the crystalline product was collected and recrystallized from ethanol/ethyl acetate (1:1 v/v) to yield 91 as yellow-green crystals (3.5 g, 13%). R$_f$ = 0.6 (light petroleum/ethyl acetate 1:1 v/v), m.p. 247-249 °C (Lit$^{54}$, m.p. 256-257 °C). $^1$H-NMR (300 MHz, [D$_6$]DMSO): (ppm) 6.85-7.02 (m, 6H), 7.26-7.40 (m, 1H), 7.67 (dd, J 7.9, 1.6 Hz, 1H), 7.85 (s, 1H), 9.85 (s, 1H). $^{13}$C-NMR (75 MHz, [D$_6$]DMSO): δ (ppm) 118.9, 119.7, 120.6, 121.2, 122.6, 122.7, 124.4, 129.7, 131.9, 133.1, 139.9, 150.3, 167.8. HRMS (ESI): m/z [M+H]$^+$ calcd. for [C$_{13}$H$_{11}$N$_2$O]$^+$ 211.0866, found: 211.0862. C$_{13}$H$_{10}$N$_2$O (210.24).

5-(2-(4-(4-Hydroxybutyl)piperidin-1-yl)acetyl)-5,10-dihydro-11H-dibenzo[b,e][1,4]diazepin-11-one (93)
4-(Piperidin-4-yl)butan-1-ol (compound 84) (1.81 g, 11.53 mmol), compound 92 (3.0 g, 10.46 mmol) and potassium carbonate (5.8 g, 42.03 mmol) were added to MeCN (80 mL) and the mixture was kept under reflux for 8 h. Insoluble material was separated by filtration and washed with CH₂Cl₂ (2 × 20 mL). The filtrate and washings were combined and the volatiles were removed under reduced pressure yielding a yellow oil, which was dissolved in CH₂Cl₂ (20 mL) followed by washing with brine. The aqueous phase was treated with CH₂Cl₂ (3 × 20 mL) and the organic extracts were collected. All organic phases were combined and dried over Na₂SO₄. Removal of the volatiles under reduced pressure gave the crude product, which was subjected to column chromatography (elucent: CH₂Cl₂/MeOH/25% aq NH₃ 96:3:1 v/v/v) to afford compound 93 as white solid (2.9 g, 62%), m.p. 143-145 °C. Rᵣ = 0.8 (CH₂Cl₂/MeOH/25% aq NH₃ 90:9:1 v/v/v). Ratio of configurational isomers evident in the NMR spectra: ca 1.2:1. ¹H-NMR (300 MHz, [D₄]MeOH): δ (ppm) 0.86-1.13 (m, 2H), 1.13-1.21 (m, 3H), 1.27-1.38 (m, 2H), 1.40-1.64 (m, 4H), 1.78-2.04 (m, 2H), 2.48-2.65 (m, 1H), 2.74-2.85 (m, 1H), 3.02 (d, J 15 Hz, 0.55H), 3.11-3.16 (m, 1H), 3.22 (d, J 15 Hz, 0.45H), 3.51 (t, J 6.5 Hz, 2H), 7.18-7.30 (m, 2H), 7.30-7.39 (m, 1H), 7.40-7.56 (m, 3H), 7.61-7.66 (m, 1H), 7.80-7.94 (m, 1H). ¹³C-NMR (75 MHz, [D₄]MeOH): δ (ppm) 24.0, 32.7, 32.9, 33.9, 36.4, 37.4, 54.8, 54.9, 62.9, 123.0, 123.1, 126.6, 127.0, 127.8, 128.9, 129.0, 129.5, 129.9, 130.6, 131.1, 132.1, 132.3, 134.3, 134.7, 135.9, 136.9, 143.7, 169.2, 169.4, 171.2, 171.5. HRMS (ESI): m/z [M+H⁺]⁺ calcd. for [C₄₂H₃₈N₃O₃⁺] 408.2282, found: 408.2299. C₂₄H₂₉N₃O₃ (407.22).

5-(2-(4-(4-Bromobutyl)piperidin-1-yl)acetyl)-5,10-dihydro-11H-dibenzo[b,e][1,4]diazepin-11-one (94)

Under an atmosphere of argon compound 93 (200 mg, 0.49 mmol) and PPh₃ (386 mg, 1.47 mmol) were dissolved in CH₂Cl₂ (5 mL) in a three-necked round bottom flask and the solution was cooled to -5 °C. A solution of CBr₄ (1.06 g, 3.20 mmol) in CH₂Cl₂ (10 mL) was added dropwise, thereby keeping the temperature of the mixture below 5 °C. Stirring was continued at room temperature overnight. The solvent was evaporated and the residue subjected to column chromatography to column chromatography (elucent: light petroleum/acetone/25% aq NH₃ 83:16:1 v/v/v) to afford compound 94 as white solid (180 mg, 78%). Rᵣ = 0.5 (light petroleum/acetone/25% aq NH₃ 66:33:1 v/v/v), m.p. 68-70 °C. Ratio of configurational isomers evident in the NMR spectra: ca 1.2:1. ¹H-NMR (300 MHz, [D₄]MeOH): δ (ppm) 1.03-1.27 (m, 5H), 1.31-1.46 (m, 2H), 1.46-1.62 (m, 2H), 1.73-1.82 (m, 2H), 1.82-2.03 (m, 2H), 2.47-2.64 (m, 1H), 2.77-2.85 (m, 1H), 3.01 (d, J 18 Hz, 0.55H), 3.10-3.18 (m, 1H), 3.21 (d, J 18 Hz, 0.45H), 3.41 (t, J 6.7 Hz, 2H), 7.17-7.40 (m, 3H), 7.40-7.59 (m, 3H), 7.61-7.66 (m, 1H), 7.84-7.90 (m, 1H). ¹³C-NMR (75 MHz, [D₄]MeOH): δ (ppm) 26.3, 32.8, 34.1, 34.4, 36.3, 36.6, 54.8, 54.9, 61.0, 122.9, 126.6, 126.9, 127.8, 128.9, 129.0, 129.5, 129.9, 130.1, 130.6, 133.0, 132.1, 134.3, 134.7, 135.9, 143.9, 169.3, 171.4. HRMS (ESI): m/z [M+H⁺]⁺ calcd. for [C₂₄H₂₉BrN₃O₂⁺]
All organic phases were combined and dried over Na$_2$SO$_4$. The aqueous phase was treated with potassium carbonate (70 mg, 0.51 mmol) and compound 28 (65 mg, 0.28 mmol) were added to MeCN (5 mL), and the mixture was kept under reflux for 3 h. Insoluble material was removed by filtration. The filtrate was concentrated under reduced pressure to yield a yellow oil, which was dissolved in CH$_2$Cl$_2$ (5 mL) followed by washing with brine. The aqueous phase was treated with CH$_2$Cl$_2$ (3 × 10 mL) and the organic extracts were collected. All organic phases were combined and dried over Na$_2$SO$_4$. Removal of the solvent under reduced pressure gave the Boc-protected intermediate as yellow oil (110 mg, 69%), which was dissolved in CH$_2$Cl$_2$/TFA/H$_2$O (10:10:1 v/v/v) (5 mL). The mixture was stirred at room temperature for 2 h. CH$_2$Cl$_2$ (10 mL) was added and the volatiles were evaporated. Purification by preparative HPLC (column: Kinetex XB C18 5 µm 250 × 21 mm; gradient: 0-30 min: MeCN/0.1% TFA 5:95-62:38; $t_R = 14$ min) afforded compound 95 as white fluffy solid (30 mg, 17%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. $^1$H-NMR (600 MHz, [D$_4$]MeOH): δ (ppm) 1.27-1.37 (m, 4H), 1.40-1.55 (m, 3H), 1.56-1.66 (m, 2H), 1.87-1.95 (m, 2H), 2.83 (s, 3H), 2.88 (t, J 7.9 Hz, 2H), 2.90-2.96 (m, 1H), 3.01-3.05 (m, 1H), 3.08-3.15 (m, 1H), 3.21-3.24 (m, 1H), 3.26-3.28 (m, 2H), 3.32-3.39 (m, 3H), 3.43-3.48 (m, 2H), 3.69-3.79 (m, 2H), 3.82-3.86 (m, 1H), 4.39 (d, J 17 Hz, 0.6H), 4.43 (d, J 17 Hz, 0.4H), 7.23-7.29 (m, 1H), 7.31-7.40 (m, 2H), 7.45-7.52 (m, 2H), 7.60-7.75 (m, 2H), 7.89 (d, J 8.0 Hz, 0.6H), 7.96 (d, J 8.0 Hz, 0.4H). $^{13}$C-NMR (150 MHz, [D$_4$]MeOH): δ (ppm) 24.8, 26.7, 30.4, 34.3, 36.4, 46.5, 52.7, 54.9, 55.3, 55.6, 56.4, 57.9, 58.0, 58.2, 59.6, 123.1, 123.7, 126.9, 127.5, 127.9, 128.5, 128.9, 129.4, 130.1, 130.5, 130.9, 131.2, 131.7, 131.9, 132.3, 132.9, 133.4, 134.6, 134.9, 135.5, 135.7, 136.9, 140.9, 142.7, 164.9, 165.5, 168.5, 168.8. RP-HPLC (220 nm): 99% ($t_R = 13.7$ min, $k = 3.8$). HRMS (ESI): $m/z$ [M+H]$^+$ calcd. for [C$_{30}$H$_{43}$N$_2$O$_2$]$^+$ 519.3442, found: 519.3441. C$_{30}$H$_{42}$N$_2$O$_2$ · C$_8$H$_4$F$_{12}$O$_6$ (518.71 + 456.09).

5-(2-(4-(4-(2-Aminoethyl)piperazin-1-yl)butyl)piperidin-1-yl)acetyl)-5,10-dihydro-11H-dibenzo[b,e][1,4]diazepin-11-one tetrakis(hydrotrifluoroacetate) (96)

Compound 94 (280 mg, 0.60 mmol), tert-butyl (2-(piperazin-1-yl)ethyl)carbamate (32) (164 mg, 0.72 mmol) and potassium carbonate (247 mg, 1.79 mmol) were added to MeCN (20 mL) and the mixture was kept under reflux for 3 h. Insoluble material was separated by filtration and washed with CH$_2$Cl$_2$ (2 × 5 mL). The filtrate and washings were combined and the solvent was evaporated. The residue was dissolved in CH$_2$Cl$_2$ (10 mL) followed by washing with brine. The aqueous phase was treated with CH$_2$Cl$_2$ (3 × 10 mL) and the organic extracts were collected. All organic phases were combined and dried over Na$_2$SO$_4$. The volatiles were removed under
reduced pressure and the residue was subjected to flash column chromatography (eluent: CH₂Cl₂/MeOH/25% aq NH₃ 90:3:1 v/v/v) to afford the Boc-protected intermediate as white solid (270 mg, 73%). Rᵣ = 0.6 (CH₂Cl₂/MeOH/25% aq NH₃ 90:10:1 v/v/v). The intermediate (270 mg, 0.436 mmol) was dissolved in CH₂Cl₂ (5 mL), TFA (1 mL) was added slowly, and the mixture was stirred at room temperature for 8 h. CH₂Cl₂ (10 mL) was added and the volatiles were evaporated. Purification by preparative HPLC (column: Kinetex XB-C18 5 µm 250 × 21 mm; gradient: 0-30 min: MeCN/0.1% aq TFA 20:80-64:36, τᵣ = 8 min) afforded compound 96 as white fluffy solid (280 mg, 66%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ¹H-NMR (600 MHz, [D₄]MeOH): δ (ppm) 1.29-1.42 (m, 4H), 1.42-1.60 (m, 3H), 1.69-1.74 (m, 2H), 1.83-2.04 (m, 2H), 2.51 (s, 2H), 2.69 (t, J 5.7 Hz, 2H), 2.84-2.99 (m, 1H), 3.00-3.24 (m, 9H), 3.38-3.60 (m, 3H), 3.70-3.80 (m, 2H), 4.39 (d, J 17 Hz, 0.65H), 4.44 (d, J 17 Hz, 0.35H), 7.24-7.29 (m, 1H), 7.31-7.38 (m, 2H), 7.45-7.52 (m, 2H), 7.60-7.75 (m, 2H), 7.89 (d, J 7.8 Hz, 0.65H), 7.96 (d, J 7.8 Hz, 0.35H). ¹³C-NMR (150 MHz, [D₄]MeOH): δ (ppm) 24.5, 24.9, 30.4, 34.3, 36.1, 37.3, 50.6, 53.0, 54.6, 54.9, 55.3, 57.7, 58.0, 123.1, 123.6, 126.8, 127.5, 127.9, 128.5, 128.9, 129.5, 130.1, 130.5, 130.9, 131.2, 131.7, 131.9, 132.3, 132.9, 133.4, 134.6, 134.9, 135.5, 135.7, 137.0, 141.0, 142.7, 164.9, 165.4, 165.8, 168.6, 168.8. RP-HPLC (220 nm): 99% (τᵣ = 13.4 min, k = 3.7). HRMS (ESI): m/z[M+H⁺]⁺ calcd. for [C₉H₁₄N₃O₂]⁺ 519.3442, found: 519.3447. C₉H₁₂N₃O₂ · C₆H₄F₁₂O₈ (518.71 + 456.09).

5-(2-(4-(4-(4-(1-Methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)propyl)piperazin-1-yl)butyl)piperidin-1-yl)acetyl)-5,10-dihydro-11H-dibenzo[b,e][1,4]diazepin-11-one tetrakis(hydrotrifluoroacetate) (97)

Compound 94 (100 mg, 0.21 mmol), compound 40 (76 mg, 0.23 mmol) and potassium carbonate (88 mg, 0.64 mmol) were added to MeCN (5 mL) and the mixture was refluxed for 6 h. Insoluble material was separated by filtration and washed with CH₂Cl₂ (2 × 10 mL). The filtrate and washings were combined and the solvent was evaporated yielding a yellow oil, which was dissolved in CH₂Cl₂ (5 mL) followed by washing with brine. The aqueous phase was treated with CH₂Cl₂ (3 × 10 mL) and the organic extracts were collected. All organic phases were combined, dried over Na₂SO₄, and the volatiles were removed under reduced pressure. Purification by preparative HPLC (column: Kinetex XB-C18 5 µm 250 × 21 mm; gradient: 0-30 min: MeCN/0.1% aq TFA 20:80-64:36, τᵣ = 8 min) afforded 97 as white fluffy solid (100 mg, 41%). Anal. calcd. for C₉₃H₇₂N₈O₃S · C₆H₄F₁₂O₈ · H₁₃O₆: C 43.89, H 5.41, N 8.71, S 2.49; found: C 43.77, H 4.63, N 8.27, S 2.35. Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ¹H-NMR (600 MHz, [D₄]MeOH): δ (ppm) 1.29-1.36 (m, 2H), 1.38-1.43 (m, 2H), 1.44-1.59 (m, 3H), 1.69-1.75 (m, 2H), 1.89-1.97 (m, 2H), 2.20-2.28 (m, 2H), 2.68-2.78 (m, 2H), 2.92-2.96 (m, 1H), 3.06 (s, 3H), 2.98-3.09 (m, 3H), 3.09-3.14 (m, 2H), 3.17-
3.30 (m, 5H), 3.42-3.45 (m, 5H), 3.63 (brs, 1H), 3.71-3.81 (m, 2H), 4.04 (d, J 14.3 Hz, 1H), 4.40 (d, J 17 Hz, 0.6H), 4.44 (d, J 17 Hz, 0.4H), 4.48-4.58 (m, 1H), 4.61 (t, J 6.2 Hz, 2H), 7.22-7.24 (m, 1H), 7.26-7.32 (m, 1H), 7.34-7.39 (m, 1H), 7.46-7.51 (m, 1H), 7.52-7.54 (m, 1H), 7.62-7.77 (m, 3H), 7.89-7.91 (m, 0.6H), 7.95-8.00 (m, 0.4H). **RP-HPLC (220 nm):** 99% (t_R = 14.2 min, k = 3.9). HRMS (ESI): m/z [M+H]^+ calcd. for [C_{39}H_{53}N_{6}O_{5}S]^+ 713.3956, found: 713.3951. C_{39}H_{52}N_{6}O_{3}S · C_{8}H_{15}F_{12}O_{8} (712.96 + 456.09).

5-(2-(4-(4-(2-Oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)-1',4'-bipiperidin)-1-y)butyl)piperidin-1-yl)acetyl)-5,10-dihydro-11H-dibenzo[b,e][1,4]diazepin-11-one tris(hydrotrifluoroacetate) (98)

Compound 98 was prepared from 94 (80 mg, 0.17 mmol), potassium carbonate (71 mg, 0.51 mmol) and 63 (56 mg, 0.19 mmol) according to the procedure for the synthesis of 97, but the reflux period was 3 h instead of 6 h. Purification by preparative HPLC (column: Kinetex XB-C18 5 μm 250 × 21 mm; gradient: 0-30 min: MeCN/0.1% aq TFA 20:80-62:38, t_R = 11 min) afforded 98 as white fluffy solid (100 mg, 57%). Ratio of configurational isomers evident in the NMR spectra: ca 1:5:1. ^1H-NMR (300 MHz, [D_4]MeOH): δ (ppm) 1.24-1.45 (m, 6H), 1.44-1.58 (m, 4H), 1.69-1.77 (m, 2H), 1.88-1.98 (m, 3H), 2.09-2.26 (m, 3H), 2.44-2.48 (m, 1H), 2.80-2.99 (m, 3H), 3.02-3.14 (m, 4H), 3.34-3.48 (m, 2H), 3.54 (t, J 6.3 Hz, 1H), 3.62-3.82 (m, 6H), 4.38-4.40 (m, 0.4H), 4.42-4.45 (m, 0.6H), 4.58-4.67 (m, 1H), 7.02-7.07 (m, 2H), 7.24-7.34 (m, 2H), 7.34-7.38 (m, 1H), 7.44-7.55 (m, 3H), 7.60-7.78 (m, 3H), 7.89-7.91 (m, 0.6H), 7.96-7.98 (m, 0.4H). ^13C-NMR (75 MHz, [D_4]MeOH): δ (ppm) 25.8, 27.8, 28.5, 29.8, 32.9, 36.4, 37.4, 52.0, 54.1, 54.9, 59.5, 61.0, 61.3, 62.8, 68.8, 110.6, 110.8, 122.2, 122.5, 123.0, 126.6, 127.0, 127.7, 128.9, 129.0, 129.4, 129.6, 129.9, 130.2, 130.5, 131.1, 132.1, 132.2, 134.3, 134.7, 136.0, 136.9, 143.7, 143.8, 156.2, 169.1, 169.3, 171.2, 171.4. RP-HPLC (220 nm): 99% (t_R = 14.9 min, k = 4.2). HRMS (ESI): m/z [M+H]^+ calcd. for [C_{41}H_{51}N_{7}O_{3}]^+ 690.4126, found: 690.4128. C_{41}H_{51}N_{7}O_{3} · C_{8}H_{15}F_{12}O_{8} (689.91 + 342.07).

1-(4-(1-(2-Oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperidin-4-yl 2,2-diphenylacetate (99)

Compound 99 was prepared from 94 (100 mg, 0.21 mmol) and 71 (69 mg, 0.23 mmol) according to the procedure for the synthesis of 97, but the reflux period was 5 h instead of 6 h. Potassium carbonate: 88 mg, 0.64 mmol. Purification by column chromatography (eluent: CH_2Cl_2/MeOH/25% aq NH_3 90:3:1 v/v/v) afforded compound 99 as white solid (40 mg, 27%).
m. p. 47-49 °C. \( R_t = 0.6 \) (CH\(_2\)Cl\(_2\)/MeOH/25% aq NH\(_3\) 90:9:1 v/v/v). Ratio of configurational isomers evident in the NMR spectra: ca 1:5:1. \(^1\)H-NMR (300 MHz, [D\(_4\)]MeOH): \( \delta \) (ppm) 0.97-1.31 (m, 8H), 1.39-1.42 (m, 2H), 1.48-1.58 (m, 1H), 1.61-1.71 (m, 2H), 1.79-2.01 (m, 4H), 2.20-2.36 (m, 4H), 2.36-2.51 (m, 2H), 2.55-2.70 (m, 1H), 2.78-2.85 (m, 1H), 2.99-3.04 (m, 0.6H), 3.11-3.26 (m, 1H), 4.84-4.87 (m, 1H), 5.07 (s, 1H), 7.21-7.25 (m, 3H), 7.28-7.32 (m, 9H), 7.36-7.40 (m, 1H), 7.41-7.51 (m, 2H), 7.53-7.56 (m, 1H), 7.61-7.66 (m, 1H), 7.84-7.90 (m, 1H). \(^{13}\)C-NMR (75 MHz, [D\(_4\)]MeOH): \( \delta \) (ppm) 25.8, 27.7, 31.0, 32.9, 33.0, 36.4, 37.4, 51.2, 54.8, 55.0, 58.4, 59.6, 71.5, 123.0, 126.6, 126.9, 127.8, 128.3, 128.9, 129.6, 129.7, 129.9, 130.6, 132.0, 132.2, 134.3, 134.7, 136.0, 136.9, 140.3, 143.8, 143.9, 169.4, 171.2, 171.5, 173.5. RP-HPLC (220 nm): 99% (\( t_k = 21.1 \) min, \( k = 6.4 \)). HRMS (ESI): \( m/z [M+H]^+ \) calcd. for [C\(_{44}H_{68}N_5O_4]^+ 685.3748, \) found: 685.3752. C\(_{44}H_{68}N_5O_4\) (684.88).

2-(4-(4-(1-(2-Oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl 9H-xanthene-9-carboxylate tris(hydrotrifluoroacetate) (100)

Compound \( 100 \) was prepared from \( 94 \) (80 mg, 0.17 mmol) and \( 77 \) (58 mg, 0.17 mmol) according to the procedure for the synthesis of \( 97 \), but the reflux period was 3 h instead of 6 h. Potassium carbonate: 94 mg, 0.68 mmol. Purification by preparative HPLC (column: Kinetex XB-C18 5 \( \mu \)m 250 × 21 mm; gradient: 0-30 min: MeCN/0.1% aq TFA 20:80-64:36, \( t_k = 16 \) min) afforded compound \( 100 \) as white fluffy solid (93 mg, 51%). Ratio of configurational isomers evident in the NMR spectra: ca 1:5:1. \(^1\)H-NMR (300 MHz, [D\(_4\)]MeOH): \( \delta \) (ppm) 1.25-1.42 (m, 4H), 1.44-1.56 (m, 3H), 1.63-1.70 (m, 2H), 1.87-2.04 (m, 2H), 2.57-2.75 (m, 5H), 2.86-3.19 (m, 8H), 3.33-3.61 (m, 2H), 3.71-3.84 (m, 2H), 4.20 (t, \( J = 5.1 \) Hz, 2H), 4.41 (d, \( J = 12 \) Hz, 0.6H), 4.47 (d, \( J = 12 \) Hz, 0.4H), 5.10 (s, 1H), 7.07-7.17 (m, 4H), 7/25-7.46 (m, 7H), 7.47-7.55 (m, 2H), 7.61-7.79 (m, 2H), 7.89-7.92 (m, 0.6 H), 7.96-7.98 (m, 0.4H). \(^{13}\)C-NMR (75 MHz, [D\(_4\)]MeOH): \( \delta \) (ppm) 19.1, 24.6, 25.1, 30.5, 34.4, 36.2, 46.6, 50.9, 42.7, 55.3, 56.7, 57.7, 58.1, 63.3, 117.9, 120.2, 122.2, 123.7, 124.6, 126.9, 127.6, 127.9, 128.6, 128.9, 130.2, 130.4, 130.5, 130.9, 131.8, 132.4, 133.5, 134.6, 136.5, 137.1, 152.9, 162.8, 165.0, 165.5, 172.9. RP-HPLC (220 nm): 99% (\( t_k = 20.3 \) min, \( k = 6.1 \)). HRMS (ESI): \( m/z [M+H]^+ \) calcd. for [C\(_{44}H_{68}N_5O_4]^+ 728.3806, \) found: 728.3805. C\(_{44}H_{68}N_5O_5\) · C\(_6\)H\(_3\)F\(_3\)O\(_6\) (727.91 + 342.07).

4-(4-(4-(1-(2-Oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)butyl 9H-xanthene-9-carboxylate (101)

Compound \( 101 \) was prepared from \( 94 \) (50 mg, 0.11 mmol) and \( 78 \) (39 mg, 0.11 mmol) according to the procedure for the synthesis of \( 97 \), but the reflux period was 5 h instead of 6 h. Potassium carbonate: 59 mg, 0.43 mmol. Purification by column chromatography (eluent: CH\(_2\)Cl\(_2\)/MeOH/25% aq NH\(_3\) 90:3:1 v/v/v) yielded compound \( 101 \) as white solid (32 mg, 38%).
m.p. 43-45 °C. R\(_f\) = 0.5 (CH\(_2\)Cl\(_2\)/MeOH/25% aq NH\(_3\) 90:9:1 v/v/v). Ratio of configurational isomers evident in the NMR spectra: ca 1:5:1. \(^1\)H-NMR (300 MHz, [D\(_3\)]MeOH): \(\delta\) (ppm) 0.98-1.11 (m, 2H), 1.24-1.37 (m, 8H), 1.41-1.64 (m, 6H), 1.84-2.00 (m, 2H), 2.15-2.25 (m, 3H), 2.26-2.38 (m, 4H), 2.38-2.51 (m, 4H), 2.61-2.65 (m, 1H), 2.78-2.85 (m, 1H), 2.99-3.04 (m, 0.6H), 3.12-3.26 (m, 1.4H), 4.04 (t, J 6.0 Hz, 2H), 5.05 (s, 1H), 7.07-7.13 (m, 4H), 7.19-7.26 (m, 2H), 7.27 (d, J 1.5 Hz, 1H), 7.30-7.38 (m, 4H), 7.41-7.49 (m, 1.6H), 7.50-7.58 (m, 1.4H), 7.62-7.67 (m, 1H), 7.84-7.91 (m, 1H). \(^{13}\)C-NMR (75 MHz, [D\(_3\)]MeOH): \(\delta\) (ppm) 23.6, 25.8, 27.6, 32.9, 33.1, 36.5, 37.5, 46.7, 53.7, 53.8, 54.9, 55.0, 58.9, 59.8, 61.1, 66.2, 117.9, 120.2, 123.1, 124.6, 126.6, 127.0, 127.8, 129.0, 129.5, 129.9, 130.2, 130.3, 132.1, 134.3, 134.7, 136.0, 136.9, 143.8, 152.8, 169.2, 169.4, 171.3, 171.5, 173.4. RP-HPLC (220 nm): 95% (\(t\_R = 19.4\) min, \(k = 5.8\)). HRMS (ESI): \(m/z\) [M+H]\(^+\) calcd. for \([C_{46}H_{54}N_6O_5]\)\(^+\) 756.4119, found: 756.4117. \(C_{46}H_{53}N_6O_5\) (755.96).

5-(2-(4-(4-((4-(1-(3-(2-Oxo-3,4-dihydroquinolin-1(2H)-yl)propyl)piperidin-4-yl)butyl)amino)butyl)piperidin-1-yl)acetyl)-5,10-dihydro-11H-dibenzo[b,e][1,4]diazepin-11-one (102)

Compound 86 (50 mg, 0.12 mmol), compound 10 (50 mg, 0.12 mmol), potassium carbonate (71 mg, 0.51 mmol) and sodium iodide (9 mg, 0.06 mmol) were added to MeCN (5 mL) and the mixture was kept under reflux for 3 h. Insoluble material was separated by filtration and washed with CH\(_2\)Cl\(_2\) (2 × 10 mL). The filtrate and washings were combined and the solvent was removed under reduced pressure yielding a yellow oily residue, which was dissolved in CH\(_2\)Cl\(_2\) (5 mL) followed by washing with brine. The aqueous phase was treated with CH\(_2\)Cl\(_2\) (3 × 10 mL) and the organic extracts were collected. All organic phases were combined, dried over Na\(_2\)SO\(_4\), and the volatiles were removed under reduced pressure. Purification by column chromatography (eluent: CH\(_2\)Cl\(_2\)/MeOH/25% aq NH\(_3\) 90:3:1 v/v/v) afforded compound 102 as yellow solid (46 mg, 52%), m.p. 141-143 °C. \(R\_f = 0.5\) (CH\(_2\)Cl\(_2\)/MeOH/25% aq NH\(_3\) 90:9:1 v/v/v). Ratio of configurational isomers evident in the NMR spectra: ca 1:5:1. \(^1\)H-NMR (300 MHz, [D\(_3\)]MeOH): \(\delta\) (ppm) 0.98-1.17 (m, 3H), 1.17-1.35 (m, 12H), 1.42-1.51 (m, 5H), 1.55-1.58 (m, 1H), 1.63-1.69 (m, 2H), 1.78-1.86 (m, 2H), 1.87-2.02 (m, 4H), 2.32-2.43 (m, 2H), 2.48-2.58 (m, 5H), 2.58-2.66 (m, 2H), 2.78-2.81 (m, 0.6H), 2.83-2.95 (m, 4H), 2.99-3.04 (m, 0.4H), 3.10-3.25 (m, 1H), 3.99 (t, J 7.3 Hz, 2H), 7.00-7.05 (m, 1H), 7.13-7.28 (m, 5H), 7.28-7.38 (m, 1H), 7.39-7.51 (m, 2H), 7.54-7.57 (m, 1H), 7.62-7.67 (m, 1H), 7.84-7.90 (m, 1H). \(^{13}\)C-NMR (75 MHz, [D\(_3\)]MeOH): \(\delta\) (ppm) 25.1, 25.4, 25.5, 26.2, 30.4, 30.7, 32.7, 32.8, 32.9, 33.0, 34.0, 36.4, 36.8, 37.4, 37.5, 41.3, 50.5, 50.6, 54.8, 55.0, 57.1, 116.4, 123.0, 124.4, 126.6, 127.0, 127.8, 128.2, 128.6, 129.0, 129.1, 129.5, 129.9, 130.6, 131.1, 131.9, 132.0, 132.2, 134.3, 135.9, 140.2, 143.8, 144.9, 169.1, 169.3, 171.2, 171.4, 172.7. RP-HPLC (220 nm): 95% (\(t\_R = 16.7\) min, \(k = 4.8\)). HRMS (ESI): \(m/z\) [M+H]\(^+\) calcd. for \([C_{45}H_{31}N_6O_3]\)\(^+\) 733.4800, found: 733.4805. \(C_{45}H_{30}N_6O_3\)
5-(2-(4-(4-(6-Amino-4-(3-((4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)propyl)-1,4-diazepan-1-yl)butyl)piperidin-1-yl)acetyl)-5,10-dihydro-11H-dibenzo[b,e][1,4]diazepin-11-one pentakis(hydrotrifluoroacetate) (105a)

Compound 94 (196 mg, 0.42 mmol), tert-butyl (1,4-diazepan-6-yl)carbamate (compound 103) (90 mg, 0.42 mmol) and compound 39 (134 mg, 0.42 mmol) were added to MeCN (10 mL), followed by the addition of potassium carbonate (116 mg, 0.84 mmol). The mixture was stirred under reflux overnight. Insoluble material was separated by filtration and washed with CH$_2$Cl$_2$ (2 × 10 mL). The filtrate and washings were combined and the solvent was evaporated to yield a yellow residue, which was dissolved in CH$_2$Cl$_2$ (5 mL) followed by washing with brine. The aqueous phase was treated with CH$_2$Cl$_2$ (3 × 10 mL) and the organic extracts were collected. All organic phases were combined, dried over Na$_2$SO$_4$, and the volatiles were removed under reduced pressure. The residue was subjected to column chromatography (eluent: CH$_2$Cl$_2$/MeOH/25%aq NH$_3$ 90:3:1) to afford the Boc-protected intermediate as colorless oil, which was dissolved in CH$_2$Cl$_2$/TFA/H$_2$O (10:10:1 v/v/v) (5 mL). The mixture was stirred at room temperature for 2 h. CH$_2$Cl$_2$ (10 mL) was added and the volatiles were evaporated. Purification by preparative HPLC (column: Kinetex XB C18 5 µm 250 × 21 mm; gradient: 0-30 min: MeCN/0.1%aq TFA 5:95-62:48, $t_f$ = 15 min) afforded compound 105a as white fluffy solid (92 mg, 17%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ¹H-NMR (600 MHz, [D$_2$]MeOH): δ (ppm) 1.27-1.41 (m, 4H), 1.41-1.47 (m, 1H), 1.49-1.58 (m, 2H), 1.65-1.79 (m, 2H), 1.84-2.01 (m, 2H), 2.07-2.20 (m, 2H), 2.64-2.85 (m, 2H), 2.92 (t, J 7.4 Hz, 3H), 3.05 (s, 3H), 2.98-3.09 (m, 3H), 3.14 (t, J 16 Hz, 2H), 3.17-3.19 (m, 1H), 3.23-3.28 (m, 1H), 3.31-3.33 (m, 1H), 3.34-3.40 (m, 1H), 3.40-3.48 (m, 2H), 3.48-3.56 (m, 2H), 3.59-3.66 (m, 1H), 3.69-3.80 (m, 2H), 3.82-3.89 (m, 1H), 3.97-4.09 (m, 1H), 4.39 (d, J 17 Hz, 0.6H), 4.43 (d, J 17 Hz, 0.4H), 4.46-4.54 (m, 1H), 4.57 (t, J 6.5 Hz, 2H), 7.20-7.23 (m, 1H), 7.23-7.30 (m, 1H), 7.29-7.42 (m, 2H), 7.44-7.56 (m, 2H), 7.61-7.76 (m, 2H), 7.89-7.90 (m, 0.6H), 7.96-7.97 (m, 0.4H). ¹³C-NMR (150 MHz, [D$_3$]MeOH): δ (ppm) 23.9, 24.6, 25.5, 27.1, 30.4, 34.3, 36.2, 43.3, 49.6, 50.9, 52.1, 53.1, 54.9, 55.3, 55.6, 56.2, 56.5, 57.9, 58.0, 59.9, 70.3, 117.0 (TFA), 118.9 (TFA), 123.1, 123.6, 125.4, 126.8, 127.5, 127.9, 128.3, 128.5, 128.9, 129.4, 130.1, 130.6, 130.9, 131.2, 131.7, 131.9, 132.4, 133.0, 133.4, 134.6, 134.9, 135.5, 135.7, 137.0, 141.0, 142.7, 145.6, 162.4 (TFA), 162.6 (TFA), 163.6, 164.9, 165.4, 168.6, 168.8. RP-HPLC (220 nm): 98% ($t_f$ = 14.3 min, $k$ = 4.0). HRMS (ESI): $m/z$ [M+H]$^+$ calc.d. for [C$_{40}$H$_{56}$N$_8$O$_3$S]$^+$ 742.4221, found: 742.42210. C$_{40}$H$_{55}$N$_9$O$_3$S · C$_{10}$H$_5$F$_{15}$O$_{10}$ (742.00 + 570.12).

$N$-(1-(3-((4-(1-Methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)propyl)-4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-
4-yl)butyl)-1,4-diazepan-6-yl)propionamide tetrakis(hydrotrifluoroacetate) (106)

Compound 105 (12.5 mg, 9.53 µmol) was dissolved in DMF (100 µL) in a 1.5-mL polypropylene reaction vessel, followed by the addition of DIPEA (17 µL, 98 µmol) and a solution of succinimidyl propionate (compound 104) (2.5 mg, 14.6 µmol) in DMF (20 µL). Stirring of the mixture was continued at room temperature for 2 h. 10% aq TFA (100 µL) was added. Purification by preparative HPLC (column: Kinetex XB-C18 5 µm 250 × 21 mm; gradient: 0-30 min: MeCN/0.1% aq TFA 5:95-62:48, t_R = 16 min) afforded compound 106 as white fluffy solid (11.4 mg, 95%). IR (KBr): 3430, 3050, 2605, 1680, 1455, 1365, 1210, 1135, 840, 725. Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. 1H-NMR (600 MHz, [D_2]MeOH): δ (ppm) 1.10 (t, J 7.6 Hz, 3H), 1.26-1.42 (m, 4H), 1.43-1.59 (m, 3H), 1.64-1.76 (m, 2H), 1.88-1.96 (m, 2H), 2.13-2.17 (m, 2H), 2.23 (q, J 7.6 Hz, 2H), 2.65-2.83 (m, 2H), 2.89-2.95 (m, 1H), 3.05 (s, 3H), 2.98-3.08 (m, 3H), 3.12-3.15 (m, 2H), 3.16-3.28 (m, 5H), 3.41-3.44 (m, 5H), 3.63 (d, J 4.5 Hz, 1H), 3.70-3.74 (m, 1.5H), 3.79 (d, J 17 Hz, 0.5H), 4.03 (d, J 15 Hz, 1H), 4.25-4.32 (m, 1H), 4.39 (d, J 17 Hz, 0.6H), 4.43 (d, J 17 Hz, 0.4H), 4.46-4.55 (m, 1H), 4.60 (t, J 6.4 Hz, 2H), 7.21-7.23 (m, 1H), 7.24-7.30 (m, 1H), 7.30-7.39 (m, 2H), 7.46-7.49 (m, 1H), 7.51-7.53 (m, 1H), 7.60-7.76 (m, 2H), 7.88-7.92 (m, 0.6H), 7.96-7.97 (m, 0.4H). 13C-NMR (150 MHz, [D_2]MeOH): δ (ppm) 10.1, 23.9, 24.5, 25.6, 26.9, 29.9, 30.4, 34.3, 36.2, 43.2, 46.9, 49.6, 50.9, 52.4, 53.1, 54.9, 55.2, 56.2, 57.5, 58.0, 58.4, 59.2, 70.1, 116.9, 123.1, 123.6, 125.4, 126.9, 127.5, 127.9, 128.4, 128.5, 128.9, 129.4, 130.1, 130.6, 130.9, 131.2, 131.7, 132.4, 133.0, 133.4, 134.6, 134.9, 135.5, 135.7, 137.1, 141.0, 142.7, 145.6, 162.1 (TFA), 162.3 (TFA), 163.6, 164.9, 165.4, 168.6, 168.8, 176.9. RP-HPLC (220 nm): 98% (t_R = 14.8 min, k = 4.2). HRMS (ESI): m/z [M+H]^+ calcd. for [C_{43}H_{58}N_{50}O_{4}S] [1,4']-5-(2-(4-(4-(6-Amino-4-(2-oxo-2-(4-(2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)-1,4'-bipiperidin)-1'-yl)ethyl)-1,4-diazepan-6-yl)butyl)piperidin-1-yl)acetyl)-5,10-dihydro-11H-dibenzo[b,e][1,4]diazepin-11-one pentakis(hydrotrifluoroacetate) (107)
temperature for 2 h. CH₂Cl₂ (10 mL) was added and the volatiles were evaporated. Purification by preparative HPLC (column: Kinetex XB-C18 5 µm 250 × 21 mm; gradient: 0-30 min: MeCN/0.1% aq TFA 5:95–62:38, tᵣ = 16 min) afforded compound 107 as white fluffy solid (19 mg, 12%). ¹H-NMR (600 MHz, [D₄]MeOH): δ (ppm) 1.36-1.42 (m, 4H), 1.42-1.59 (m, 3H), 1.64-1.72 (m, 1H), 1.73-1.87 (m, 3H), 1.89-1.97 (m, 2H), 2.09-2.22 (m, 4H), 2.69 (t, J 13 Hz, 1H), 2.80-2.89 (m, 2H), 2.91-2.97 (m, 1H), 3.00-3.07 (m, 2H), 3.07-3.22 (m, 4H), 3.24-3.27 (m, 3H), 3.33-3.41 (m, 1H), 3.41-3.49 (m, 2H), 3.50-3.68 (m, 4H), 3.69-3.76 (m, 5H), 3.76-3.83 (m, 2H), 3.96-3.98 (m, 1H), 4.39 (d, J 17 Hz, 0.6H), 4.43 (d, J 17 Hz, 0.4H), 4.57-4.62 (m, 1H), 4.72-4.74 (m, 1H), 7.047.07 (m, 3H), 7.22-7.30 (m, 1H), 7.29-7.38 (m, 3H), 7.46-7.49 (m, 1H), 7.50-7.54 (m, 1H), 7.61-7.64 (m, 1H), 7.66-7.76 (m, 1H), 7.89 (d, J 7.7 Hz 0.6H), 7.96 (d, J 7.7 Hz, 0.4H). ¹³C-NMR (150 MHz, [D₄]MeOH): δ (ppm) 24.5, 25.0, 27.3, 27.5, 27.8, 27.9, 30.4, 34.3, 41.6, 43.9, 50.2, 50.4, 51.8, 54.9, 55.3, 56.1, 58.0, 58.5, 59.9, 64.7, 109.9, 110.7, 115.0 (TFA), 116.9 (TFA), 118.9 (TFA), 120.9 (TFA), 122.4, 122.9, 123.1, 123.6, 126.8, 127.5, 127.9, 128.5, 128.9, 129.4, 129.7, 130.1, 130.5, 130.9, 131.2, 131.7, 131.9, 132.3, 133.0, 133.4, 134.6, 134.9, 135.5, 135.7, 137.0, 141.0, 142.7, 156.1, 162.3 (TFA), 162.5 (TFA), 162.8 (TFA), 162.9 (TFA), 164.4, 164.5, 168.6, 168.8, 170.8. RP-HPLC (220 nm): 99% (tᵣ = 14.9 min, k = 4.2). HRMS (ESI): m/z [M+2H]⁺ calc. for [C₄₈H₆₄N₁₀O₄]⁴⁺ 423.2629, found: 423.2613. C₄₈H₆₄N₁₀O₄·C₁₀H₅F₁₅O₁₀ (845.11 + 570.12).

**N-(1-(4-(1-(2-Oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)-4-((2-oxo-2-(4-(2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)-1',4'-bipiperidin]-1'-yl)ethyl)-1,4-diazepan-6-yl)propionamide tetrakis(hydrotrefluoracetaet) (108)**

Compound 108 was prepared from 107 (7.6 mg, 5.37 µmol) and 104 (1.4 mg, 8.18 µmol) according to the procedure for the synthesis of 106. DIPEA: 10 µL, 58 µmol. Purification by preparative HPLC (column: Kinetex XB-C18 5 µm 250 × 21 mm; gradient: 0-30 min: MeCN/0.1% aq TFA 5.95-62.48, tᵣ = 16 min) yielded compound 108 as hygroscopic white fluffy solid (7 mg, 96%). Ratio of isomers evident in the NMR spectra: ca 1.5:1. ¹H-NMR (600 MHz, [D₄]MeOH): δ (ppm) 1.14 (t, J 7.6 Hz, 3H), 1.32-1.45 (m, 4H), 1.45-1.59 (m, 3H), 1.60-1.80 (m, 4H), 1.80-1.89 (m, 1H), 1.91-1.99 (m, 2H), 2.09-2.12 (m, 2H), 2.19-2.24 (m, 2H), 2.27 (q, J 12 Hz, 2H), 2.68-2.76 (m, 1H), 2.81-2.89 (m, 2H), 2.89-3.00 (m, 2H), 3.03-3.17 (m, 4H), 3.16-3.23 (m, 3H), 3.33-3.51 (m, 5H), 3.51-3.60 (m, 2H), 3.69-3.85 (m, 6H), 3.98-4.06 (m, 1H), 4.12-4.19 (m, 1H), 4.41 (d, J 17 Hz, 0.6H), 4.45 (d, J 17 Hz, 0.4H), 4.58-4.62 (m, 1H), 4.74-4.76 (m, 1H), 7.05-7.08 (m, 3H), 7.19-7.40 (m, 4H), 7.47-7.51 (m, 1H), 7.51-7.53 (m, 1H), 7.63 (dd, J 15, 7.1 Hz, 1H), 7.66-7.76 (m, 1H), 7.90 (d, J 8.0 Hz, 0.6H), 7.97 (d, J 8.0 Hz, 0.4H). ¹³C-NMR (150 MHz, [D₄]MeOH): δ (ppm) 10.2, 24.5, 25.4, 27.3, 27.5, 27.9, 30.1, 30.4, 34.3, 36.2, 41.7, 44.1, 50.2, 50.4, 54.9, 55.3, 56.7, 57.2, 58.0, 59.2, 59.6, 61.0, 64.8, 109.9, 110.7, 116.9 (TFA), 118.9
(TFA), 122.4, 122.9, 123.1, 123.6, 126.9, 127.5, 127.9, 128.5, 128.9, 129.4, 129.7, 130.1,
130.6, 130.9, 131.2, 131.7, 131.9, 132.4, 133.0, 133.4, 134.6, 134.9, 135.5, 135.7, 137.0,
141.0, 142.7, 156.1, 162.6 (TFA), 162.8 (TFA), 164.9, 165.5, 168.6, 168.8, 177.1. RP-HPLC
(220 nm): 99% (tR = 15.6 min, k = 4.4). HRMS (ESI): m/z [M+2H]2+ calcd. for [C51H70N10O5]2+

5-(Aminomethyl)-N1-(4-((4-(1-methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-
3-yl)oxy)pentyl)amino)-4-oxobutylnyl)isophthalamide (110) and

5-(Aminomethyl)-N1,N3-bis(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-
dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-
yl)ethyl)isophthalamide heptakis(hydrotrifluoroacetate) (112)

TBTU (244 mg, 0.76 mmol) and DIPEA (131 μL, 0.76 mmol) were added to a solution of 109
(113 mg, 0.38 mmol) and HOBT (103 mg, 0.76 mmol) in DMF (2 mL) and the mixture
was stirred at room temperature for 20 min. A solution of 52 (140 mg, 0.38 mmol), 96 (370 mg,
0.38 mmol) and DIPEA (131 μL, 0.76 mmol) in DMF (2 mL) was added dropwise and stirring
was continued at 60 °C for 3 h. H2O (10 mL) was added followed by extraction with ethyl
acetate (3 × 5 mL). The combined extracts were dried over Na2SO4 and concentrated under
reduced pressure to yield the Boc-protected intermediate as yellow oil, which was dissolved
in CH2Cl2/TFA/H2O (10:10:1 v/v/v) (5 mL). The mixture was stirred at room temperature for 2 h.
CH2Cl2 (10 mL) was added and the volatiles were evaporated. Purification by preparative
HPLC (column: Kinetex XB-C18 5 μm 250 × 21 mm; gradient: 0-25 min: MeCN/0.1% aq TFA
12:88-64:36, tR (112) = 11 min, tR (110) = 12 min) afforded compound 110 (101 mg, 14%) and
compound 112 (60 mg, 8%) as white fluffy solids. 110: ratio of isomers evident in the NMR
spectra: ca 1:5:1. 1H-NMR (600 MHz, [D4]MeOH): δ (ppm) 1.23-1.43 (m, 5H), 1.43-1.54 (m,
5H), 1.54-1.63 (m, 3H), 1.68-1.73 (m, 2H), 1.82-2.01 (m, 7H), 2.28 (t, J 7.5 Hz, 2H), 2.69-2.81
(m, 2H), 2.89-2.95 (m, 4H), 3.02-3.05 (m, 1H), 3.05 (s, 3H), 3.06-3.11 (m, 3H), 3.15-3.22 (m,
3H), 3.31-3.39 (m, 3H), 3.39-3.47 (m, 4H), 3.58-3.68 (m, 3H), 3.70-3.73 (m, 1.5H), 3.78 (d, J
18 Hz, 0.5H), 4.02-4.05 (m, 1H), 4.23 (s, 2H), 4.39 (d, J 17 Hz, 0.6H), 4.43 (d, J 17 Hz, 0.4H),
4.51 (t, J 6.5 Hz, 2H), 7.23-7.26 (m, 1H), 7.27-7.39 (m, 2H), 7.46-7.53 (m, 2H), 7.59-7.76 (m,
3H), 7.89-7.90 (m, 0.6 H), 7.95-7.96 (m, 0.4H), 8.04-8.10 (m, 2H), 8.29-8.30 (m, 1H). 13C-NMR
(150 MHz, [D4]MeOH): δ (ppm) 22.5, 23.0, 23.1, 23.8, 25.4, 28.1, 28.7, 29.0, 32.9, 33.2, 34.7,
36.1, 38.8, 39.3, 41.9, 42.4, 48.2, 49.6, 50.9, 51.8, 53.5, 53.9, 55.7, 56.3, 56.6, 70.9, 121.7,
122.2, 124.1, 125.5, 126.1, 126.3, 126.5, 126.8, 127.1, 127.5, 127.5, 128.0, 128.7, 129.2,
129.5, 129.8, 130.3, 130.6, 130.9, 131.6, 132.0, 133.2, 133.5, 134.1, 134.2, 134.3, 135.4,
135.6, 135.8, 139.5, 139.6, 141.3, 144.2, 160.2 (TFA), 160.4 (TFA), 160.6 (TFA), 160.8 (TFA),
162.4, 163.6, 164.0, 167.1, 167.2, 167.4, 167.6, 174.0. RP-HPLC (220 nm): 96% (t_R = 14.9 min, k = 4.2). HRMS (ESI): m/z [M+H]^+ calcd. for [C_{65}H_{77}N_{12}O_{6}S]^+ 1045.5810, found: 1045.5803. C_{65}H_{76}N_{12}O_{6}S · C_{10}H_{15}F_{15}O_{10} (1045.36 + 570.12).

112: Ratio of isomers evident in the NMR spectra: ca 1.5:1. 1H-NMR (600 MHz, [D_4]MeOH): δ (ppm) 1.29-1.42 (m, 8H), 1.41-1.60 (m, 6H), 1.63-1.77 (m, 4H), 1.88-1.96 (m, 4H), 2.83-2.97 (m, 2H), 2.98-3.10 (m, 6H), 3.11-3.14 (m, 4H), 3.16-3.28 (m, 4H), 3.30-3.36 (m, 4H), 3.26-3.60 (m, 10H), 3.69-3.72 (m, 6H), 3.77-3.89 (m, 2H), 4.23 (s, 2H), 4.40 (d, J 17 Hz, 1.2H), 4.44 (d, J 17 Hz, 0.8H), 7.21-7.35 (m, 4H), 7.36-7.38 (m, 1H), 7.44-7.54 (m, 4H), 7.58-7.71 (m, 4H), 7.55-7.73 (m, 1H), 7.86-7.91 (m, 1.2H), 7.95-7.96 (m, 0.8H), 8.10 (s, 2H), 8.34 (s, 1H). 13C-NMR (150 MHz, [D_4]MeOH): δ (ppm) 23.1, 23.7, 29.0, 32.9, 34.7, 35.6, 42.4, 49.3, 50.1, 53.5, 53.9, 56.0, 56.3, 56.6, 113.7 (TFA), 115.6 (TFA), 117.6 (TFA), 119.5 (TFA), 121.7, 122.3, 125.5, 126.1, 126.5, 127.1, 127.5, 128.1, 128.7, 129.1, 129.5, 129.8, 130.3, 130.6, 130.8, 130.9, 131.6, 132.0, 133.2, 133.5, 134.1, 134.3, 135.2, 135.6, 139.6, 141.3, 161.0 (TFA), 161.4 (TFA), 163.6, 164.1, 167.2, 167.4, 167.7. RP-HPLC (220 nm): 98% (t_R = 14.4 min, k = 4.1). HRMS (ESI): m/z [M+H]^+ calcd. for [C_{65}H_{78}N_{13}O_{6}]^{2+} 589.8602, found: 589.8601. C_{65}H_{78}N_{13}O_{6} · C_{14}H_{7}F_{15}O_{14} (1196.56 + 798.16).

N^1-(4-((4-(1-Methyl-1,2,5,6-tetrahydropyridin-3-yl)-1,2,5-thiadiazol-3-yl)oxy)pentyl)amino)-4-oxobutyl)-N^2-(2-(4-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butoxy(piperazin-1-yl)ethyl)-5-(propionamidomethyl)isophthalamid tetraakis(hydrotrifluoroacetate) (111)

Compound 111 was prepared from 110 (16 mg, 8.7 μmol) and 104 (2.3 mg, 13 μmol) according to the procedure for the synthesis of 106. DIPEA: 17 μL, 98 μmol. Purification by preparative HPLC (column: Kinetex XB-C18 5 μm 250 × 21 mm; gradient: 0-25 min: MeCN/0.1% aq TFA 20:80-95:5, t_R = 9 min), yielded compound 111 as white fluffy solid (12 mg, 89%). Ratio of isomers evident in the NMR spectra: ca 1.5:1. 1H-NMR (600 MHz, [D_4]MeOH): δ (ppm) 1.14 (t, J 7.6 Hz, 3H), 1.31-1.43 (m, 6H), 1.43-1.61 (m, 7H), 1.64-1.79 (m, 2H), 1.84-1.94 (m, 6H), 1.92-1.99 (m, 1H), 2.24-2.30 (m, 4H), 2.61-2.68 (m, 1H), 2.69-2.83 (m, 3H), 2.85-2.97 (m, 4H), 2.99-3.14 (m, 9H), 3.18 (t, J 6.6 Hz, 2H), 3.39-3.41 (m, 3H), 3.42-3.53 (m, 1H), 3.61 (t, J 6.2 Hz, 3H), 3.70-3.80 (m, 2H), 4.03 (d, J 15 Hz, 1H), 4.39 (d, J 17 Hz, 0.6H), 4.41 (d, J 17 Hz, 0.4H), 4.44 (d, J 4.5 Hz, 2H), 4.50 (t, J 6.5 Hz, 2H), 7.23-7.24 (m, 1H), 7.26-7.40 (m, 2H), 7.46-7.48 (m, 1H), 7.50-7.53 (m, 1H), 7.59-7.68 (m, 2H), 7.68-7.76 (m, 1H), 7.88 (s, 2H), 7.89-7.90 (m, 0.6H), 7.96-7.97 (m, 0.4H), 8.14-8.15 (m, 1H). 13C-NMR (150 MHz, [D_4]MeOH): δ (ppm) 10.4, 23.9, 24.4, 24.5, 25.2, 26.5, 26.8, 29.5, 30.0, 30.1, 30.4, 34.3, 34.6, 36.1, 37.5, 40.2, 40.6, 43.3, 43.7, 51.0, 52.3, 53.2, 54.9, 55.3, 57.4, 57.8, 58.0, 72.4, 115.1 (TFA), 116.4 (TFA), 117.0 (TFA), 118.9 (TFA), 123.1, 123.6, 125.4, 126.1, 126.9, 127.5, 127.9, 128.2, 128.5, 128.9, 129.4, 130.1, 130.3, 130.6, 130.9, 131.2, 131.7, 132.3, 133.0, 133.4, 134.6, 134.9, 135.5,
136.7, 136.2, 136.5, 137.0, 141.0, 141.5, 142.7, 145.6, 163.8, 164.9, 165.4, 168.6, 168.8, 169.2, 169.6, 171.1, 171.9, 175.4, 177.1. RP-HPLC (220 nm): 98% (t<sub>R</sub> = 15.9 min, k = 4.5).

HRMS (ESI): m/z [M+H]<sup>+</sup> calcd. for [C<sub>59</sub>H<sub>80</sub>N<sub>12</sub>O<sub>7</sub>S]<sup>+</sup> 1101.6072, found: 1101.6066. C<sub>59</sub>H<sub>80</sub>N<sub>12</sub>O<sub>7</sub>S · C<sub>8</sub>H<sub>6</sub>F<sub>12</sub>O<sub>6</sub> (1101.43 + 456.09).

**N'<sub>1</sub>,N'<sub>2</sub>-Bis-(2-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)(ethyl)-5-(propionamidomethyl)isophthalamide hexakis(hydrotrifluoroacetate) (113)**

Compound 113 was prepared from 112 (17 mg, 8.52 µmol) and 104 (2.3 mg, 13 µmol) according to the procedure for the synthesis of 106. DIPEA: 16 µL, 93 µmol. Purification by preparative HPLC (column: Kinetex XB-C18 5 µm 250 × 21 mm; gradient: 0-25 min: MeCN/0.1% aq TFA 20:80-64.36, t<sub>R</sub> = 11 min), yielded compound 113 as white fluffy solid (13 mg, 79%).

Ratio of isomers evident in the NMR spectra: ca 1.5:1. ¹H-NMR (600 MHz, [D<sub>6</sub>]-MeOH): δ (ppm) 1.14 (t, J 7.6 Hz, 3H), 1.30-1.48 (m, 10H), 1.48-1.61 (m, 4H), 1.68-1.79 (m, 4H), 1.85-2.01 (m, 4H), 2.28 (q, J 7.6 Hz, 2H), 2.81-2.99 (m, 4H), 2.99-3.08 (m, 6H), 3.08-3.17 (m, 6H), 3.17-3.26 (m, 4H), 3.34-3.48 (m, 8H), 3.48-3.65 (m, 4H), 3.38-3.83 (m, 6H), 4.36-4.51 (m, 4H), 7.20-7.31 (m, 2H), 7.32-7.35 (m, 2H), 7.36-7.39 (m, 1H), 7.44-7.51 (m, 2H), 7.51-7.53 (m, 2H), 7.61-7.69 (m, 4H), 7.72-7.78 (m, 1H), 7.89 (d, J 7.7 Hz, 1.2H), 7.93 (s, 2H), 7.96 (d, J 7.7 Hz, 0.8H), 8.25 (s, 1H). ¹³C-NMR (150 MHz, [D<sub>6</sub>]-MeOH): δ (ppm) 10.4, 24.5, 25.1, 30.1, 30.4, 34.2, 36.1, 37.2, 43.5, 50.8, 52.0, 54.9, 55.3, 57.5, 57.8, 58.1, 116.9 (TFA), 118.9 (TFA), 123.1, 123.7, 126.3, 126.9, 127.5, 127.9, 128.5, 128.9, 129.5, 130.1, 130.5, 130.7, 130.9, 131.2, 131.7, 131.9, 132.3, 133.0, 133.4, 134.6, 134.9, 135.5, 135.7, 136.0, 137.0, 141.0, 141.6, 142.7, 162.4 (TFA), 162.6 (TFA), 165.0, 165.5, 168.6, 168.8, 169.6, 177.1. RP-HPLC (220 nm): 95% (t<sub>R</sub> = 15.3 min, k = 4.3). HRMS (ESI): m/z [M+H]<sup>+</sup> calcd. for [C<sub>72</sub>H<sub>94</sub>N<sub>13</sub>O<sub>7</sub>S]<sup>+</sup> 1252.7394, found: 1252.7375. C<sub>72</sub>H<sub>93</sub>N<sub>13</sub>O<sub>7</sub> · C<sub>12</sub>H<sub>6</sub>F<sub>18</sub>O<sub>12</sub> (1252.62 + 684.14).

**5-(Aminomethyl)-N'<sub>1</sub>-(2-(4-(2-oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)-1,4'-bipiperidin)-1'-yl)ethyl)-N'<sub>2</sub>-(2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)isophthalamide hexakis(hydrotrifluoroacetate) (114)**

Compound 114 was prepared from 109 (80 mg, 0.27 mmol), 96 (263 mg, 0.27 mmol) and 64 (93 mg, 0.27 mmol) according to the procedure for the synthesis of 110 and 112. TBTU: 173 mg, 0.54 mmol; HOBt: 73 mg, 0.54 mmol; DIPEA: 189 + 189 µL, 1.1 + 1.1 mmol. Purification by preparative HPLC (column: Kinetex XB-C18 5 µm 250 × 21 mm; gradient: 0-25 min: MeCN/0.1% aq TFA 15:85-64:36, t<sub>R</sub> (112) = 10 min, t<sub>R</sub> (114) = 12 min) yielded compounds 112 (25 mg, 5%) and 114 (45 mg, 10%) as white fluffy solids. 114: ratio of isomers evident in the NMR spectra: ca 1.5:1. ¹H-NMR (600 MHz, [D<sub>6</sub>]-MeOH) δ (ppm) 1.31-1.42 (m, 4H), 1.43-1.59
N¹-(2-(4-(2-Oxo-2,3-dihydro-1H-benzo[d]imidazol-1-yl)-[1,4'-bipiperidin]-1'-yl)ethyl)-N²-(2-(4-(4-(1-(11-oxo-10,11-dihydro-5H-dibenzo[b,e]1,4-diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)-5-(propionamidomethyl)isophthalamide pentakis(hydo trifluoroacetate) (115)

Compound 115 was prepared from 114 (20 mg, 11.7 μmol) and 104 (3.2 mg, 18.7 μmol) according to the procedure for the synthesis of 106. DIPEA: 22 μL, 130 μmol. Purification by preparative HPLC (column: Kinetex XB-C18 5 μm 250 × 21 mm; gradient: 0-25 min: MeCN/0.1% aq TFA 20:80-64:36, tR = 9 min), yielded compound 115 as white fluffy solid (17 mg, 88%). IR (KBr): 3400, 3070, 2960, 1750, 1515, 1485, 1460, 1360, 1380, 1295, 1135, 835, 800, 720. Ratio of isomers evident in the NMR spectra: ca 1.5:1. ¹H-NMR (600 MHz, [D₆]DMSO) δ (ppm) 1.02 (t, J 7.6 Hz, 3H), 1.15-1.29 (m, 4H), 1.31-1.47 (m, 3H), 1.53-1.66 (m, 2H), 1.73-1.80 (m, 2H), 1.87-2.05 (m, 4H), 2.16 (q, J 7.6 Hz, 2H), 2.34-2.38 (m, 2H), 2.60-2.72 (m, 2H), 2.75-3.07 (m, 3H), 2.97-3.05 (m, 4H), 3.05-3.31 (m, 4H), 3.32-3.38 (m, 3H), 3.46-3.55 (m, 4H), 3.55-3.62 (m, 4H), 3.65-3.70 (m, 2H), 3.73-3.97 (m, 3H), 3.83 (d, J 5.9 Hz, 2H), 4.39 (d, J 7.6 Hz, 2H), 4.43 (d, J 7.6 Hz, 0.4H), 4.60 (t, J 12 Hz, 1H), 6.93-7.02 (m, 3H), 7.22-7.30 (m, 2H), 7.33-7.35 (m, 1H), 7.42-7.47 (m, 1H), 7.50-7.60 (m, 1H), 7.68-7.77 (m, 2H), 7.80-7.82 (m, 1H), 7.86-7.88 (m, 0.4H), 7.88 (s, 2H), 8.21 (s, 1H), 8.41-8.43 (m, 1H), 8.75 (brs, 1H), 8.91 (brs, 1H), 9.61 (brs, 0.6H), 10.66 (brs, 0.4H), 10.73 (s, 0.4H), 10.78 (s, 0.6H), 10.94 (s, 1H). ¹³C-NMR (150 MHz, [D₆]DMSO) δ (ppm) 9.9, 23.0, 23.5, 23.7, 23.8, 25.4, 25.8, 28.5, 28.6, 32.7, 34.4, 34.8, 35.5, 40.1, 41.8, 46.5, 48.4, 49.0, 50.4, 52.7, 53.2, 54.9, 55.9, 59.5, 108.5, 109.1, 113.6, 115.6, 117.6, 119.5, 120.5, 120.9, 122.3, 124.7, 124.9, 125.5, 127.3, 128.8, 130.0, 130.1, 130.5, 130.9, 131.2, 132.7, 131.9, 132.3, 132.4, 132.9, 133.4, 134.6, 134.9, 135.4, 135.5, 135.7, 136.1, 136.7, 137.0, 140.9, 142.7, 156.1, 162.3 (TFA), 162.6 (TFA), 162.8 (TFA), 163.0 (TFA), 164.9, 165.5, 168.6, 168.8, 168.9, 169.4. RP-HPLC (220 nm): 98% (tR = 14.6 min, k = 4.1). HRMS (ESI): m/z [M+H]⁺ calcd. for [C₅₈H₇₇N₁₂O₃]⁺ 1021.6140, found: 1021.6140. C₅₈H₇₇N₁₂O₅ · C₁₂H₄F₁₈O₁₂ (1021.33 + 684.14).
127.7, 128.3, 128.4, 128.7, 128.9, 129.0, 129.7, 130.0, 130.4, 131.0, 131.6, 133.0, 133.1, 133.8, 134.1, 134.4, 134.7, 135.8, 139.5, 140.4, 141.0, 153.6, 158.2 (TFA), 158.4 (TFA), 158.6 (TFA), 158.8 (TFA), 164.2, 165.7, 166.1, 166.4, 170.3, 173.1. RP-HPLC (220 nm): 99% \( (t_r = 15.0 \text{ min}, k = 4.2) \). HRMS (ESI): \( [M+H]^+ \) calcd. for \( \text{[C}_{61}\text{H}_{61}\text{N}_{12}\text{O}_{6}]^+ \) 1077.6397, found: 1077.6392. \( \text{C}_{61}\text{H}_{60}\text{N}_{12}\text{O}_{6} \cdot \text{C}_{10}\text{H}_{5}\text{F}_{15}\text{O}_{10} \) (1077.39 + 570.12).

1-(2-(3-(Aminomethyl)-5-yl)-(2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzof[2,1]dihydro-5H-[1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)carbamoyl)benzamido)ethyl)piperidin-4-yl)butyl)piperazin-1-yl]ethyl)carbamoyl)benzamido)ethyl)piperidin-4-yl carboxylate (116)

Compound 116 was prepared from 109 (80 mg, 0.27 mmol), 96 (262 mg, 0.27 mmol) and 73 (92 mg, 0.27 mmol) according to the procedure for the synthesis of 110 and 112. TBTU: 173 mg, 0.54 mmol; HOBt: 73 mg, 0.54 mmol; DIPEA: 95 + 95 μL, 0.54 + 0.54 mmol. Purification by preparative HPLC (column: Kinetex XB-C18 5 μm 250 × 21 mm; gradient: 0-25 min: MeCN/0.1% aq TFA 20:80-95:5, \( t_r \) (112) = 9 min, \( t_r \) (116) = 11 min) afforded compounds 112 (30 mg, 6%) and 116 (120 mg, 28%) as white fluffy solids. 116: ratio of configurational isomers evident in the NMR spectra: ca 1:5.1. 1H-NMR (600 MHz, [D₄]MeOH): \( \delta \) (ppm) 1.26-1.40 (m, 4H), 1.40-1.58 (m, 3H), 1.68-1.73 (m, 2H), 1.81-1.99 (m, 3H), 2.05-2.14 (m, 2H), 2.25-2.26 (m, 1H), 2.78-2.96 (m, 2H), 2.99 (t, \( J \) 6.7 Hz, 2H), 3.03-3.05 (m, 1H), 3.06-3.10 (m, 2H), 3.11-3.22 (m, 3H), 3.23-3.26 (m, 2H), 3.39-3.46 (m, 6H), 3.55-3.57 (m, 2H), 3.66 (t, \( J \) 6.2 Hz, 2H), 3.69-3.88 (m, 5H), 4.23 (s, 2H), 4.39 (d, \( J \) 17 Hz, 0.6H), 4.43 (d, \( J \) 17 Hz, 0.4H), 5.04-5.09 (m, 1H), 5.18 (d, \( J \) 18 Hz, 1H), 7.25-7.28 (m, 4H), 7.31-7.38 (m, 8H), 7.45-7.49 (m, 1H), 7.51 (d, \( J \) 7.8 Hz, 1H), 7.56-7.81 (m, 3H), 7.88-7.90 (m, 0.6H), 7.95-7.96 (M, 0.4H), 8.11-8.13 (m, 2H), 8.36 (s, 1H). 13C-NMR (150 MHz, [D₄]MeOH): \( \delta \) (ppm) 24.5, 25.1, 28.1, 28.9, 30.4, 34.3, 35.8, 36.1, 37.3, 43.8, 49.6, 50.9, 51.9, 54.9, 55.3, 57.4, 57.8, 58.0, 58.1, 66.0, 115.1, 116.9, 117.1 (TFA), 118.9 (TFA), 123.1, 123.7, 126.9, 127.5, 127.8, 127.9, 128.5, 128.9, 129.5, 129.7, 130.1, 130.5, 130.9, 131.2, 131.7, 131.9, 132.2, 132.3, 132.4, 133.0, 133.4, 134.6, 134.9, 135.4, 135.7, 136.1, 136.7, 137.0, 140.0, 141.0, 142.7, 158.8, 159.1, 162.4 (TFA), 162.6 (TFA), 162.8 (TFA), 163.1 (TFA), 164.9, 165.4, 168.6, 168.8, 168.9, 169.4. RP-HPLC (220 nm): 99% \( (t_r = 17.9 \text{ min}, k = 5.2) \). HRMS (ESI): \( [M+H]^+ \) calcd. for \( \text{[C}_{60}\text{H}_{74}\text{N}_{9}\text{O}_{6}]^+ \) 1016.5757, found: 1016.5750. \( \text{C}_{60}\text{H}_{73}\text{N}_{9}\text{O}_{6} \cdot \text{C}_{10}\text{H}_{5}\text{F}_{15}\text{O}_{10} \) (1016.30 + 570.12).

1-(2-(3-(2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzof[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)carbamoyl)benzamido)ethyl)piperidin-4-yl)-(propionamidomethyl)benzamido)ethyl)piperidin-4-yl carboxylate (117)

Compound 117 was prepared from 116 (15 mg, 9.5 μmol) and 104 (2.9 mg, 16.9 μmol)
according to the procedure for the synthesis of 106. DIPEA: 16 µL, 92 µmol. Purification by preparative HPLC (column: Kinetex XB-C18 5 µm 250 × 21 mm; gradient: 0-25 min: MeCN/0.1% aq TFA 20:80-95:5, \( t_\text{R} = 11 \text{ min} \)), afforded compound 117 as hygroscopic white fluffy solid (12.1 mg, 83%). Anal. calcd. for \( \text{C}_{63}\text{H}_{77}\text{N}_{6}\text{O}_{7} \cdot \text{C}_{6}\text{H}_{12}\text{F}_{8} \cdot \text{H}_{14}\text{O}_{7} \): C 51.54, H 5.79, N 7.62; found: C 51.61, H 5.27, N 7.26. Ratio of isomers evident in the NMR spectra: ca 1.5:1. \(^1\)H-NMR (600 MHz, [D\(_4\)]MeOH): \( \delta \) (ppm) 1.14 (t, J 7.6 Hz, 3H), 1.30-1.41 (m, 4H), 1.43-1.57 (m, 3H), 1.68-1.73 (m, 2H), 1.83-1.96 (m, 3H), 2.03-2.13 (m, 2H), 2.27 (q, J 7.6 Hz, 2H), 2.80-2.89 (m, 2H), 2.90-2.94 (m, 3H), 2.98-3.02 (m, 2H), 3.05-3.11 (m, 3H), 3.12-3.19 (m, 2H), 3.23-3.26 (m, 2H), 3.34-3.37 (m, 4H), 3.40-3.48 (m, 2H), 3.55-3.57 (m, 1H), 3.63 (t, J 6.2 Hz, 2H), 3.67-3.85 (m, 5H), 4.36-4.42 (m, 1H), 4.44 (s, 2H), 5.04-5.19 (m, 2H), 7.24-7.28 (m, 3H), 7.29-7.39 (m, 10H), 7.46-7.53 (m, 2H), 7.61-7.64 (m, 1H), 7.65-7.76 (m, 1H), 7.89-7.90 (m, 0.6H), 7.92 (d, J 9.3 Hz, 2H), 7.96-7.97 (m, 0.4H), 8.21 (s, 1H). \(^{13}\)C-NMR (150 MHz, [D\(_4\)]MeOH): \( \delta \) (ppm) 10.4, 24.5, 25.2, 28.2, 29.0, 30.1, 30.4, 34.3, 35.9, 36.1, 37.4, 43.7, 49.6, 50.9, 52.1, 54.9, 55.3, 57.5, 57.8, 58.1, 65.9, 116.9 (TFA), 118.9 (TFA), 123.1, 123.6, 126.4, 126.9, 127.5, 127.9, 128.5, 128.9, 129.4, 129.7, 130.1, 130.5, 130.6, 130.8, 130.9, 131.9, 132.4, 133.0, 133.4, 134.6, 134.9, 135.5, 135.7, 136.2, 137.0, 140.0, 141.0, 141.7, 142.7, 162.0 (TFA), 162.3 (TFA), 162.5 (TFA), 162.8 (TFA), 164.9, 165.4, 165.6, 168.6, 168.8, 169.5, 170.1, 177.1. RP-HPLC (220 nm): 98% (\( \text{t}_\text{R} = 18.9 \text{ min, } k = 5.6 \)). HRMS (ESI): \( m/z \left[ \text{M}+\text{H}\right]^+ \) calcd. for \[\text{C}_{63}\text{H}_{77}\text{N}_{6}\text{O}_{7}\]^+ 1072.6024, found: 1072.6013. \( \text{C}_{63}\text{H}_{77}\text{N}_{6}\text{O}_{7} \cdot \text{C}_{6}\text{H}_{12}\text{F}_{8} \) (1072.37 + 456.09).

2-(4-(2-(3-(Aminomethyl))-5-(2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)carbamoyl)benzamido)ethyl)piperazin-1-yl)ethyl \( 9H \)-xanthene-9-carboxylate hexakis(hydrotrifluoroacetate) (118)

Compound 118 was prepared from 109 (80 mg, 0.27 mmol), 96 (262 mg, 0.27 mmol) and 80 (102 mg, 0.27 mmol) according to the procedure for the synthesis of 110 and 112. TBTU: 172 mg, 0.54 mmol; HOBt: 73 mg, 0.54 mmol; DIPEA: 95 + 95 µL, 0.55 + 0.55 mmol. Purification by preparative HPLC (column: Kinetex XB-C18 5 µm 250 × 21 mm; gradient: 0-25 min: MeCN/0.1% aq TFA 20:80-95:5, \( \text{t}_\text{R} = 11 \text{ min} \)) afforded compounds 112 (20 mg, 4%) and 118 (70 mg, 15%) as white fluffy solids. 118: ratio of isomers evident in the NMR spectra: ca 1.5:1. \(^1\)H-NMR (600 MHz, [D\(_4\)]MeOH): \( \delta \) (ppm) 1.29-1.39 (m, 5H), 1.41-1.60 (m, 3H), 1.67-1.72 (m, 2H), 1.83-2.00 (m, 2H), 2.78 (t, J 4.8 Hz, 2H), 2.69-2.72 (m, 4H), 2.97 (t, J 6.2 Hz, 2H), 3.03-3.12 (m, 6H), 3.15-3.25 (m, 4H), 3.30-3.51 (m, 8H), 3.66 (t, J 6.3 Hz, 2H), 3.68-3.82 (m, 4H), 4.20-4.24 (m, 2H), 4.25 (brs, 2H), 4.39 (d, J 17 Hz, 0.6H), 4.43 (d, J 17 Hz, 0.4H), 5.10 (s, 1H), 7.07-7.17 (m, 4H), 7.23-7.30 (m, 1H), 7.31-7.39 (m, 6H), 7.46-7.53 (m, 2H), 7.61-7.65 (m, 1H), 7.66-7.71 (m, 0.6H), 7.73-7.76 (m, 0.4H), 7.89-7.90 (m, 0.6H), 7.94-7.99 (m, 0.4H), 8.10-8.18 (m, 2H), 8.38-8.39 (m, 1H). \(^{13}\)C-NMR (150 MHz, [D\(_4\)]MeOH): \( \delta \)
(ppm) 23.1, 23.8, 29.0, 32.9, 34.7, 35.0, 35.9, 42.4, 45.2, 49.5, 50.1, 50.6, 51.3, 53.5, 53.9, 55.1, 55.9, 56.0, 56.4, 56.6, 61.7, 113.5 (TFA), 115.5 (TFA), 116.4, 117.4 (TFA), 118.7, 119.3 (TFA), 121.7, 122.2, 123.3, 125.5, 126.1, 126.4, 126.5, 127.1, 127.5, 128.0, 128.7, 129.0, 129.1, 129.5, 129.8, 130.3, 130.6, 130.8, 130.9, 131.0, 131.6, 132.0, 133.2, 133.5, 134.1, 134.2, 134.3, 135.0, 135.4, 135.6, 139.6, 141.3, 151.5, 160.8 (TFA), 161.0 (TFA), 161.3 (TFA), 163.5, 164.0, 167.2, 167.4, 167.5, 167.8, 171.3. RP-HPLC (220 nm): 96% (tR = 17.9 min, k = 5.2). HRMS (ESI): m/z [M+H]+ calcd. for [C61H75N10O7]+ 1059.5815, found: 1059.5796. C61H74N10O7 ⋅ C12H8F18O12 (1059.33 + 684.14).

2-(4-(2-(3-((2-(4-(1-(2-Oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)carbamoyl)-5-(propionamidomethyl)benzamido)ethyl)piperazin-1-yl)ethyl 9H-xantheine-9-carboxylate pentakis(hydrotrifluoroacetate) (119)

Compound 119 was prepared from 118 (16 mg, 9.18 µmol) and 104 (2.3 mg, 13.4 µmol) according to the procedure for the synthesis of 106. DIPEA: 16 µL, 92 µmol. Purification by preparative HPLC (column: Kinetex XB-C18 5 µm 250 × 21 mm; gradient: 0-25 min: MeCN/0.1% aq TFA 20:80-95:5, tR = 10 min), yielded compound 118 as hygroscopic white fluffy solid (13.3 mg, 86%). Ratio of isomers evident in the NMR spectra: ca 1:5.1. 1H-NMR (600 MHz, [D6]MeOH): δ (ppm) 1.14 (t, J 7.6 Hz, 3H), 1.27-1.41 (m, 5H), 1.41-1.57 (m, 3H), 1.67-1.72 (m, 2H), 1.88-1.95 (m, 2H), 2.28 (q, J 7.6 Hz, 2H), 2.65-2.71 (m, 4H), 2.78-2.83 (m, 2H), 2.81-2.97 (m, 2H), 2.96-2.98 (m, 3H), 3.01-3.09 (m, 6H), 3.12-3.25 (m, 5H), 3.35-3.45 (m, 4H), 3.65 (t, J 6.6 Hz, 2H), 3.69-3.72 (m, 3H), 3.73-3.81 (m, 1H), 4.18-4.25 (m, 2H), 4.39 (d, J 17 Hz, 0.6H), 4.43 (d, J 17 Hz, 0.4H), 4.46 (s, 2H), 5.10 (s, 1H), 7.10-7.14 (m, 4H), 7.24-7.30 (m, 1H), 7.30-7.34 (m, 3H), 7.34-7.41 (m, 3H), 7.45-7.50 (m, 1H), 7.49-7.53 (m, 1H), 7.59-7.66 (m, 1H), 7.66-7.71 (m, 0.6H), 7.73-7.76 (m, 0.4H), 7.89 (m, 0.6H), 7.93-7.97 (m, 2.4H), 8.23-8.24 (m, 1H). 13C-NMR (150 MHz, [D6]MeOH): δ (ppm) 10.4, 24.5, 25.1, 30.1, 30.4, 34.3, 36.1, 36.4, 37.3, 43.7, 46.6, 50.9, 51.5, 52.0, 52.7, 54.9, 55.3, 56.5, 57.4, 57.5, 57.8, 58.0, 63.1, 116.9 (TFA), 117.9, 118.8 (TFA), 120.1, 123.1, 123.6, 124.6, 126.4, 126.9, 127.5, 127.9, 128.5, 128.9, 129.4, 130.1, 130.4, 130.5, 130.6, 130.7, 130.9, 131.2, 131.7, 132.0, 132.4, 133.0, 133.4, 134.6, 134.9, 135.5, 135.7, 136.1, 137.0, 141.0, 141.7, 142.7, 152.9, 162.1 (TFA), 162.4 (TFA), 162.6 (TFA), 164.9, 165.4, 168.6, 168.8, 169.6, 169.9, 172.7, 177.2. RP-HPLC (220 nm): 96% (tR = 18.9 min, k = 5.6). HRMS (ESI): m/z [M+H]+ calcd. for [C64H78N10O8]+ 1115.6082, found: 1115.6076. C64H78N10O8 ⋅ C10H6F15O10 (1115.39 + 570.12).

5-(Aminomethyl)-N\(^1\)-(2-(4-(1-(2-Oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)-N\(^2\)-(2-(4-(1-(3-(2-oxo-3,4-dihydroquinolin-1(2H)-yl)propyl)piperidin-4-yl)butyl)piperazin-1-
yl)ethyl]isophthalamide heptakis(hydrotrifluoroacetate) (120)

Compound 120 was prepared from 109 (80 mg, 0.27 mmol), 96 (263 mg, 0.27 mmol) and 88 (123 mg, 0.27 mmol) according to the procedure for the synthesis of 110 and 112. TBTU: 172 mg, 0.54 mmol; HOBt: 73 mg, 0.54 mmol; DIPEA: 94 + 94 μL, 0.54 + 0.54 mmol. Purification by preparative HPLC (Kinetex XB-C18 5 μm 250 × 21 mm; gradient: 0-20 min: MeCN/0.1% aq TFA 10:90-35:65, \( t_R \) (112) = 18.5 min, \( t_R \) (120) = 19.1 min) afforded compounds 112 (15 mg, 3%) and 120 (22 mg, 4%) as white fluffy solids. 120: ratio of isomers evident in the NMR spectra: ca 1.5:1.

\[ ^1HNMR \text{ (600 MHz, [D}^4\text{]MeOH): } \delta \text{ (ppm) } 1.30-1.41 (m, 10H), 1.48-1.62 (m, 4H), 1.69-1.79 (m, 4H), 1.89-1.92 (m, 1H), 1.93-1.98 (m, 2H), 2.06-2.14 (m, 2H), 2.59-2.69 (m, 2H), 2.89-2.95 (m, 5H), 3.00-3.09 (m, 6H), 3.14-3.18 (m, 7H), 3.20-3.27 (m, 5H), 3.37-3.49 (m, 8H), 3.54-3.59 (m, 4H), 7.04-7.06 (m, 1H), 7.17-7.23 (m, 2H), 7.24-7.39 (m, 4H), 7.46-7.49 (m, 1H), 7.50-7.53 (m, 1H), 7.61-7.64 (m, 1H), 7.67-7.76 (m, 1H), 7.89 (d, \( J = 7.5 \text{ Hz}, 0.6H), 7.96 (d, \( J = 7.6 \text{ Hz}, 0.4H), 8.12 (s, 2H), 8.38 (s, 1H). \]

\[ ^13CNMR \text{ (150 MHz, [D}^4\text{]MeOH): } \delta \text{ (ppm) } 23.7, 24.5, 25.1, 26.1, 30.4, 30.7, 32.6, 34.3, 34.5, 36.0, 36.1, 36.2, 37.1, 37.2, 40.2, 43.8, 49.6, 50.8, 51.7, 51.8, 54.1, 54.9, 55.0, 55.3, 55.7, 57.4, 57.8, 57.9, 58.1, 61.0, 116.1, 116.9 (TFA), 117.5, 118.1 (TFA), 118.9 (TFA), 123.1, 123.7, 124.8, 126.9, 127.5, 127.9, 128.2, 128.5, 128.8, 128.9, 129.3, 129.5, 130.1, 130.5, 130.9, 131.2, 131.7, 131.9, 132.2, 132.3, 133.0, 133.4, 134.6, 134.9, 135.4, 135.5, 135.7, 136.6, 137.0, 139.6, 141.0, 142.7, 162.0 (TFA), 162.3 (TFA), 162.6 (TFA), 164.9, 165.5, 168.6, 168.8, 168.9, 173.3. RP-HPLC (220 nm): 98% (\( t_R = 15.4 \text{ min}, k = 4.4 \). HRMS (ESI): \( m/z [M+H]^+ \) calcd. for \( [C_{66}H_{93}N_{12}O_5]^{+} \) 1133.7392, found: 1133.7386.

\[ C_{66}H_{92}N_{12}O_5 \cdot C_{14}H_7F_{21}O_{14} (1133.54 + 798.16). \]

3.4.2. Synthesis of the radioligands \([^3H]106\) and \([^3H]115\)

The tritiated heterodimeric ligands \([^3H]106\) and \([^3H]115\) were prepared by \([^3H]propionylation\) of the precursor amines 105a and 114, respectively. A solution of succinimidyl [2,3-\(^3H\)]-proprionate (specific activity: 80 Ci/mmol, purchased from American Radiolabeled Chemicals, St. Louis, MO, via Hartman Analytics, Braunschweig, Germany) (2.5 mCi, 5.5 μg, 31.25 nmol (each)) in hexane/EtOAc (9:1) was transferred from the delivered ampoule to a 1.5-mL reaction vessel with screw cap, and the solvent was removed in a vacuum concentrator (ca 30 min at about 30 °C). A solution of the precursor molecule (105a: 0.53 mg, 403 nmol; 114: 0.52 mg, 305 nmol) in anhydrous DMF/DIPEA (50:1 v/v) (60 μL) was added, and the vessel was vigorously shaken at rt for 1.5 h. 2% aq TFA (40 μL) and MeCN/H₂O (10:90 v/v) (300 μL) were added and the radioligands were purified using an analytical HPLC system (Waters, Eschborn, Germany) consisting of two 510 pumps, a pump control module, a 486 UV/vis detector, and a Flow-one Beta series A-500 radiodetector (Packard, Meriden, CT). A Luna C18
(3 μm, 150 mm × 4.6 mm, Phenomenex, Aschaffenburg, Germany) was used as stationary phase at a flow rate of 0.8 mL/min. Mixtures of 0.05% aq TFA (A) and acetonitrile containing 0.04% TFA (B) were used as mobile phase. The following linear gradient was applied: 0-20 min: A/B 90:10-79:21, 20-25 min: 79:21 (isocratic), 25-27 min: 79:21-5:95, 27-35 min: 5:95. For the purification of each radioligand three HPLC runs were performed (UV detection: 220 nm; no radiometric detection). Each radioligand was collected in a 2-mL reaction vessel with screw cap (t_{R} ([\text{^3}H]106) = 25.0 min, t_{R} ([\text{^3}H]115) = 25.2 min). The volume of the combined eluates was reduced in a vacuum concentrator to approx. 400 μL and approx. 300 µL, respectively and ethanol (400 and 300 µL, respectively) was added. The solutions were transferred into 3-mL borosilicate glass vials with conical bottom (Wheaton NextGen 3-mL vials). The reaction vessels were rinsed twice with EtOH/water (50:50 v/v) (200 and 300 µL, respectively) and the washings were transferred to the 3-mL glass vials to obtain tentative stocks with volumes of 1200 µL. For the quantification of the radioligands, a four-point calibration was performed with the corresponding ‘cold’ forms 106 (0.1, 0.2, 0.5, and 0.8 μM) and 115 (0.1, 0.2, 0.5, and 1 µM) using the following HPLC conditions: HPLC system, stationary phase, eluents and flow rate as above; linear gradient for [\text{^3}H]106: 0-20 min: A/B 90:10-69:31, 20-22 min: 69:31-5:95, 22-29 min: 5:95; linear gradient for [\text{^3}H]115: 0-20 min: A/B 90:10-72:28, 20-22 min: 72:28-5:95, 22-29 min: 5:95; injection volume: 100 µL; UV detection: 220 nm. A 2-µL aliquot of each tentative radioligand stock solutions was added to 128 µL of acetonitrile/0.05% aq TFA (10:90 v/v), 100 µL of this solution were analyzed by HPLC, and five times 2 µL were counted in 3 mL of scintillator (Rotiszint eco plus; Carl Roth, Karlsruhe, Germany) with a LS 6500 liquid scintillation counter (Beckmann-Coulter, Munich, Germany). These analyses were performed twice. The molarities of the tentative stock solutions of [\text{^3}H]106 and [\text{^3}H]115 were calculated from the mean of the peak areas and the linear calibration curves obtained from the peak areas of the standards. To determine the radiochemical purities and to prove the chemical identities, solutions (100 µL) of [\text{^3}H]106 (0.18 μM) and [\text{^3}H]115 (0.23 μM) spiked with 106 (3 μM) and 115 (3 μM), respectively, were analyzed by RP-HPLC using the system, column, eluents, flow rate, injection volume and UV detection as for the quantification and additionally radiometric detection (flow rate of the liquid scintillator (Rotiscint eco plus/acetonitrile (90:10 v/v)): 4.0 mL/min) The following linear gradient was used: 0-20 min: A/B 90:10-69:31, 20-30 min: 69:31-5:95, 30-38 min: 5:95). The radiochemical purities amounted to 98% and 99%, respectively. The analyses were repeated after storage at -20 °C for 10 months and revealed radiochemical purities of 88% and 98%, respectively. Calculated specific activities: [\text{^3}H]106, 2.420 TBq/mmol (65.40 Ci/mmol), [\text{^3}H]115, 1.815 TBq/mmol (49.06 Ci/mmol). The final activity concentrations were adjusted to 18.50 MBq/mL by the addition of EtOH/water (50:50 v/v), resulting in molarities of 7.64 μM ([\text{^3}H]106) and 10.2 μM ([\text{^3}H]115). Radiochemical yields: [\text{^3}H]106, 33.64 MBq, 36%; [\text{^3}H]115, 32.56 MBq, 35%.
3.4.3. Investigation of the chemical stability

The chemical stability of 106 and 115 was investigated in PBS (pH = 7.4) at 22 ± 1 °C. The incubation was started by addition of a 10 mM solution of the compounds in DMSO (1 μL) to PBS (99 μL) to give a final concentration of 100 μM. After 0, 12, and 48 h, an aliquot (20 μL) was taken and added to acetonitrile/0.04% aq TFA (1:9 v/v) (20 μL). An aliquot (20 μL) of the resulting solution was analyzed by RP-HPLC using a system from Agilent Technologies (composed of a 1290 Infinity binary pump equipped with a degasser, a 1290 Infinity autosampler, a 1290 Infinity thermostated column compartment, a 1260 Infinity diode array detector, and a 1260 Infinity fluorescence detector). A Kinetex-XB C18 2.6 μm, 100 × 3 mm (Phenomenex) served as stationary phase at a flow rate of 0.5 mL/min. The following linear gradient was applied: 0-20 min: 0.04% aq TFA/acetonitrile 10:90-68:32, 20-22 min: 68:32-95:5, 22-28 min: 95:5. The detection wavelength was set to 220 nm.

3.4.4. Cell culture and preparation of cell homogenates

CHO-K9 cell lines stably transfected with the human M1-M5 muscarinic receptors were obtained from Missouri S&T cDNA Resource Center (Rolla, MO). Cells were cultured in HAM’s F12 medium supplemented with fetal calf serum (Biochrom, Berlin, Germany) (10%) and G418 (Biochrom) (750 µg/mL). CHO-hM2 cell homogenates were prepared according to a reported procedure with minor modifications70: the harvest buffer (50 mM TRIS, 1 mM EDTA) was supplemented with protease inhibitor (SIGMAFAST, Sigma-Aldrich)). Aliquots of 200 μL were transferred to 2-mL cups and stored at -80°C.

3.4.5. MR radioligand binding experiments

All radioligand binding experiments were performed at 22 ± 1 °C. Leibovitz L-15 medium (Gibco, Life Technologies GmbH, Darmstadt, Germany) supplemented with 1% BSA (Serva, Heidelberg, Germany) (in the following referred to as L15 medium) was used as binding buffer throughout. The effects of various MR ligands on the equilibrium binding of [3H]NMS (equilibrium competition binding assay) were determined at intact adherent CHO-hM,R cells (x = 1-5) in white 96-well plates with clear bottom (Corning Life Sciences, Tewksbury, MA; Corning cat. no. 3610) using the protocol of previously described MR binding studies with [3H]NMS55 with the following modification: the total volume of L15 medium per well was 200 μL instead of 188 μL, i.e. the cells were covered with L15 medium (160 μL) followed by the
addition of L15 medium (20 µL), neat or containing atropine 10-fold concentrated, and L15 medium (20 µL) containing the radioligand 10-fold concentrated. The concentration of \(^{3}H\)NMS was 0.2 nM (M1, M2, M3), 0.1 nM (M4) or 0.3 nM (M5) and the incubation time was 3 h throughout.

Saturation binding experiments with \(^{3}H\)106 and \(^{3}H\)115 at live adherent CHO-hM2 cells were also performed as previously described binding studies with \(^{3}H\)NMS\(^{55}\). The incubation period was 2 h. Nonspecific binding was determined in the presence of atropine (7) (500-fold excess to the radioligand).

Saturation binding experiments with \(^{3}H\)106 and \(^{3}H\)115 at CHO-hM2 cell homogenates and the investigation of the effects of various MR ligands on equilibrium binding of \(^{3}H\)106 and \(^{3}H\)115 (equilibrium competition binding assay), investigated at CHO-hM2 cell homogenates, too, were performed in Primaria 96-well plates (Corning Life Sciences) using a final volume of 100 µL per well. On the day of the experiment, CHO-hM2 cell homogenates were thawed and re-suspended using a 1-mL syringe (Henke-Sass Wolf GmBh, Tuttlingen, Germany) equipped with a needle (0.90 × 40 mm, B. Braun, Melsungen, Germany) followed by centrifugation at 500 g at 4°C for 5 min. The supernatant was discarded and the pellets were re-suspended in L15 medium using a 1-mL syringe equipped with a needle (0.45 × 25 mm, B. Braun). The homogenates were stored on ice until use. The total amount of protein per well was between 19 and 43 µg. Wells were prefilled with 70 µL of L15 medium. For total binding, L15 medium (10 µL), L15 medium (10 µL) containing the radioligand 10-fold concentrated and cell homogenate (10 µL) were added. To determine unspecific binding or the effect of a compound of interest on radioligand equilibrium binding, L15 medium (10 µL) containing atropine 10-fold concentrated (500-fold excess to the radioligand) or the compound of interest (competitor) 10-fold concentrated, L15 medium (10 µL) containing the radioligand 10-fold concentrated and cell homogenate (10 µL) were added. The applied radioigand concentrations for competition binding studies were 2.0 nM (\(^{3}H\)106) and 0.3 nM (\(^{3}H\)115). The plates were shaken during incubation (2 h for saturation and competition binding experiments). After the incubation the homogenates were collected on GF/C filter mats (0.26 mm; Whatman, Maidstone, UK) (pretreated with 0.3% aq polyethyleneimine) and washed with cold PBS using a Brandel Harvester (Brandel, Gaithersburg, MD). Filter pieces for each well were punched out and transferred into 1450-401 96-well plates (PerkinElmer). Rotiscint eco plus (Carl Roth) (200 µL) was added, the plates were sealed with a transparent sealing tape (permanent seal for microplates, PerkinElmer, prod. no. 1450–461), vigorously shaken for at least 3 h, and afterwards kept in the dark for at least 1 h prior to the measurement of radioactivity (DPM) with a MicroBeta2 plate counter (PerkinElmer, Rodgau, Germany). In case of saturation binding experiments performed with \(^{3}H\)115 in the presence of 15 (applied at increasing fixed concentrations), the total volume per well was 200 µL. Wells were prefilled with 130 µL of L15
medium. For total binding, L15 medium (20 µL), L15 medium (20 µL) containing 15 10-fold concentrated, L15 medium (20 µL) containing the radioligand 10-fold concentrated and cell homogenate (10 µL) were added. To determine unspecific binding, L15 medium (20 µL) containing atropine 10-fold concentrated (500-fold excess to [³H]115), L15 medium (20 µL) containing 15 10-fold concentrated, L15 medium (20 µL) containing the radioligand 10-fold concentrated and cell homogenate (10 µL) were added.

M₂R association and dissociation experiments with [³H]106 and [³H]115 were performed at CHO-hM₂ cell homogenates in Primaria 96-well plates (Corning Life Sciences) using the experimental procedure as for saturation and competition binding experiments at CHO-hM₂ cell homogenates (see above). The concentration of [³H]106 and [³H]115 used for association experiments was 2 nM and 0.6 nM, respectively. The incubation was started after different periods of time (between 0 and 120 min) and stopped immediately after the last addition of radioligand by collecting and washing the homogenates on GF/C filter mats using the harvester. Unspecific binding was determined in the presence of 7 (500-fold excess to the radioligand). In case of dissociation experiments a preincubation of the cell homogenates with the radioligand ([³H]106: 4 nM, [³H]115: 0.6 nM) was performed for 60 min. The preincubation was started after different periods of time ([³H]106: between 1 and 180 min, [³H]115: between 0 and 150 min) by the addition of the radioligand to the wells prefilled with L15 medium and cell homogenates. The dissociation was started by addition of 7 (1000-fold excess to the radioligand) dissolved in L15 medium (10 µL) (10-fold concentrated). The dissociation was stopped by collecting and washing the homogenates using the harvester. For the determination of unspecific binding 7 (1000-fold excess to the radioligand) was added during the preincubation step.

3.4.6. IP1 accumulation assay

The measurement of M₂R stimulated activation of the G-protein mediated pathway was performed applying the IP-One HTRF® assay (Cisbio, Codolet, France) according to the manufacturer’s protocol. In brief, HEK-293 cells were grown to a confluence of approx. 70% and transiently co-transfected with the cDNAs of the human M₂ receptor (Missouri S&T cDNA Resource Center) and the hybrid G-protein Gₐq5-HA (Gₐq protein with the last five amino acids at the C-terminus replaced by the corresponding sequence of Gₐi; gift from the J. David Gladstone Institutes, San Francisco, CA)⁷¹-⁷² applying TransIT-293 Mirus transfection reagent (MoBiTec, Goettingen, Germany). After one day cells were detached from the culture dish with Versene (Life Technologies GmbH, Darmstadt, Germany), seeded into black 384-well plates (10,000 cells/well) (Greiner Bio-One, Frickenhausen, Germany) and maintained for 24 h at 37 °C. After incubation with the test compounds dissolved in stimulation buffer (final concentration
range from 1 pM up to 100 µM) at 37 °C for 1 h the detection reagents were added (IP1-d2 conjugate and Anti-IP1cryptate TB conjugate each dissolved in lysis buffer), and incubation was continued at room temperature for 60 min. Time resolved fluorescence resonance energy transfer (HTRF) was determined using the Clariostar plate reader (BMG, Ortenberg, Germany) measuring fluorescence at 620 (± 10) nm and 670 (± 10) nm (excitation at 330 nm). In the agonist mode each compound (106, 115) was tested in duplicate in three individual experiments in comparison to the reference compound carbachol (1, eight experiments). Antagonist properties of 7, 106 and 115 were determined after preincubation of the cells with 7, 106 or 115 for 30 min, subsequent addition of the MR agonist 1 (at a final concentration of 300 nM) and continued incubation at 37 °C for 1 h (five independent experiments each).

3.5. Data processing

Retention (capacity) factors were calculated from retention times (tR) according to k = (tR−t0)/t0 (t0 = dead time). Data of the IP1 accumulation assay (agonist mode) were processed by plotting the ratios (emission 670 nm/emission 620 nm) of the HTRF measurements against log(concentration 1) and analysis by a four-parameter logistic equation (GraphPad Prism Software 6.0, GraphPad Software, San Diego, CA), followed by normalization (0% = ‘top’ (maximum of IP1 accumulation), 100% = ‘bottom’ (basal activity)) of the four-parameter logistic fit and analysis of the normalized data by a four-parameter logistic equation (log(agonist) vs. response - variable slope). Data of the IP1 accumulation assay (antagonist mode) were processed by plotting the fluorescence ratio against log(concentration antagonist) and analysis by a four-parameter logistic equation (GraphPad Prism), followed by normalization (0% = ‘top’ (IP1 accumulation elicited by 1 (0.3 µM)) of the four-parameter logistic fit, 100% = ‘bottom’ (basal activity)) and analysis of the normalized data by a four-parameter logistic equation (log(inhibitor) vs. response - variable slope). pIC50 values were converted into pKb values according to the Cheng-Prusoff equation73 (logarithmic form). Specific binding data (DPM) from saturation binding experiments were plotted against the free radioligand concentration and analyzed by a two-parameter equation describing hyperbolic binding (one site-specific binding, GraphPad Prism) to obtain Kd and Bmax values. The free radioligand concentration (nM) was calculated by subtracting the amount of specifically bound radioligand (nM) (calculated from the specifically bound radioligand in dpm, the specific activity and the volume per well) from the total radioligand concentration per well. Unspecific binding data from saturation binding experiments were fitted by linear regression. In case of saturation binding experiments with [3H]115 in the presence of compound 15, specific binding data (in DPM) were additionally normalized to the Bmax value and specific binding (%) was plotted against
log(concentration $[^3]H$115) followed by analysis using a four-parameter logistic fit (log(agonist) vs. response, applied constraints: bottom = 0%, top = 100%; GraphPad Prism) (cf. Figure 10A). Data for the ‘Schild’ analysis were obtained from the rightward shift ($\Delta pK_d$) of the saturation isotherm and transformation into log(r-1) (where $r = 10^{\Delta pK_d}$). Log(r-1) was plotted against log(concentration 15) and the data were analyzed by linear regression to obtain the slope and the ‘pA2’ value (intercept with the X axis). Specific binding data from association experiments with $[^3]H$106 and $[^3]H$115 were analyzed by a two-parameter equation describing an exponential rise to a maximum (one-phase association, GraphPad Prism) to obtain the observed association rate constant $k_{obs}$ and the maximum of specifically bound radioligand ($B_{eq}$), which was used to calculate specifically bound radioligand ($B_i$) in %. Data from dissociation experiments (% specifically bound radioligand ($B_i$) plotted over time) were analyzed by a three-parameter equation (one phase decay, GraphPad Prism) (in case of $[^3]H$106 ‘plateau’ was defined as 0) to obtain the dissociation rate constant $k_{off}$. The association rate constants ($k_{on}$) were calculated from $k_{obs}$, $k_{off}$ and the radioligand concentration ([RL]) according to the correlation: $k_{on} = (k_{obs}-k_{off})/[RL]$. Total binding data (DPM) from radioligand competition binding experiments (determination of the effect of various MR ligands on the equilibrium binding of $[^3]H$NMS, $[^3]H$106 or $[^3]H$115) were plotted against log(concentration competitor) and analyzed by a four-parameter logistic equation (log(inhibitor) vs. response-variable slope, GraphPad Prism) followed by normalization (100% = ‘top’ of the four-parameter logistic fit, 0% = unspecifically bound radioligand (DPM) in case of using $[^3]H$NMS, or 0% = ‘bottom’ of the four-parameter logistic in case of using $[^3]H$106 and $[^3]H$115) and analysis of the normalized data by a four-parameter logistic equation. IC$_{50}$ were converted to $K_i$ values according to the Cheng-Prusoff equation using $K_d$ values of 1.1 nM ($[^3]H$106) and 0.12 nM ($[^3]H$115)$^{73}$. Statistical significance was assessed by a one-sample t-test. Propagated errors were calculated according to the Gaussian law of errors.

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Chapter 4

Dibenzodiazepinone-type fluoroscently labeled muscarinic receptor ligands
4. Dibenzodiazepinone-type fluorescently labeled muscarinic receptor ligands

4.1. Introduction

Fluorescent GPCR ligands are considered as useful molecular tools complementary to radioligands for studying GPCRs. Compared with radioligands, fluorescent ligands are advantageous, for instance, with respect to safety issues and high costs for disposal. Moreover, appropriate fluorescent ligands can serve to study the localization of receptors in cells by fluorescence microscopy, and to probe the geometry and mechanisms of ligand-receptor interactions and functional responses at a single cell\(^1\). Typically, fluorescent ligands are composed of a pharmacophore (a known agonist or antagonist for the receptor of interest), a linker and the fluorophore\(^2\). To design fluorescent ligands with receptor binding properties comparable to the parent ligand, several factors need to be taken into consideration (in particular in case of low molecular weight/non-peptide ligands): the attachment site and length of the linker as well as the type of the fluorophore (size, lipophilicity, net charge, etc.)\(^3\) can be crucial. Numerous fluorescent ligands for GPCRs have been reported, for example probes for NPY\(^4\)-\(^8\), histamine\(^9\)-\(^13\), opioid\(^14\)-\(^16\), dopamine\(^17\) and muscarinic receptors\(^18\)-\(^26\).

Muscarinic acetylcholine receptors (MRs), in humans constituting five subtypes (M\(_1\)R-M\(_5\)R), are widely distributed in both the peripheral and central nervous systems\(^27\)-\(^29\), and are involved in the regulation of various physiological functions. The development of MR ligands, which bind with high selectivity to one of the five subtypes, proved to be highly challenging due to the high conservation of the orthosteric binding site among MRs. As allosteric binding sites are less conserved, the design of allosterically or dualsterically binding MR ligands is considered a promising approach to develop ligands with higher subtype selectivity\(^30\)-\(^31\). For instance, improved MR subtype binding or functional selectivity was reported for dualsterically/bitopically interacting antagonists such as pirenzepine derivatives\(^32\)-\(^34\) and the dimeric compound methoctramine\(^35\), as well as for agonists such as McN-A-343\(^36\) and derivatives of AC-42\(^21\), \(^37\).

Various fluorescent MR antagonists such as telenzepine conjugated to Eosin-5 or Cascade Blue dyes\(^38\)-\(^39\), and pirenzepine labeled with Bodipy FL\(^40\) were used to study the distribution and expression of M\(_1\) receptors in cultured neurons derived from rat visual cortex or presented as alternatives to radiotracers. A series of fluorescent ligands derived from the M\(_1\)R-prefering antagonist pirenzepine were synthesized by Ilien and coworkers\(^23\), \(^41\). The fluorophores were linked with pirenzepine through linkers of varying chain length. Among these compounds, derivative Bo(15)PZ (compound 121, cf. Figure 1) was suggested to bind M\(_1\)R in a bitopic
manner, addressing the orthosteric site (via the pirenzepine moiety) and additionally a brucine accessible allosteric site. Compound 122 (para-LRB-AC42, cf. Figure 1), representing a derivative of the bitopic agonist AC-42 labeled with lissamine rhodamine B, inhibited orthosteric [³H]NMS binding at the M₁R under equilibrium conditions, and was suggested to be competitive with the allosteric modulator 14 (cf. Chapter 3). A putative bitopic/dualsteric binding pose for 122 at the human M₁ receptor was supported by molecular modeling studies. In contrast to the M₁R, reports on fluorescent M₂-M₅ receptor ligands are rare.

The observation that dibenzodiazepinone-type heterodimeric ligands, composed of the dibenzodiazepinone scaffold (DIBA, compound 9), a linker and a second (varying) MR pharmacophore, exhibited throughout high M₂R affinity (Kᵢ < 10 nM) (cf. Chapter 3), stimulated us to prepare fluorescently labeled ligands derived from 9, a high affinity M₂R antagonist. As the bulky 'side chain' (comprising the linker and the second pharmacophoric group) in DIBA-derived dimeric ligands was well tolerated with respect to M₂R binding, we anticipated that also bulky fluorophores attached to 9 will not or only marginally affect binding to the M₂R. As the radiolabeled homo- and heterodimeric dibenzodiazepinone-type MR ligands [³H]11 and [³H]115, respectively (cf. doctoral thesis of Andrea Pegoli and Chapter 3), were shown to bind dualsterically to the M₂R, the fluorescent ligands presented in this chapter were supposed to exhibit a dualsteric binding mode, too, and were characterized in this respect.

Seven DIBA-derived fluorescent MR ligands were synthesized by linking red-fluorescent fluorophores (cf. Figure 2) to the dibenzodiazepinone scaffold using different types of linkers. MR binding data were determined by equilibrium competition binding with [³H]NMS. Two selected fluorescent ligands were characterized by flow cytometry- and high content imaging-based binding studies (saturation and competition binding). Moreover, MR binding was visualized using confocal microscopy.

**Figure 1.** Structures of the fluorescently labeled MR ligands 121 and 122, which were suggested to exhibit a bitopic/dualsteric binding mode at the M₁ receptor.
4.2. Results and discussion

4.2.1. Chemistry

The fluorescent dibenzodiazepinone-type MR ligands were prepared using red fluorescent dyes (emission wavelength > 590 nm) in order to have low background fluorescence when applying the fluorescent ligands at cells. Three different Cy5-related cyanine dyes, i.e. S0223, S0436 and S0387 (emission maximum > 650 nm) (cf. Figure 2) as well as the pyrylium dye Py-5 (emission maximum > 600 nm) (cf. Figure 2) were used to prepare the fluorescent ligands. The cyanine dyes S0223, S0436 and S0387, exhibiting a low Stokes' shift, can be excited at 635 nm with a red diode laser. Pyrylium dyes such as Py-5, originally developed for the staining of proteins\(^ {43}\), react readily with primary amines at pH > 8 to give the corresponding pyridinium adducts (cf. Scheme 1), which are characteristic of a large Stokes’ shift and can be excited with an argon laser (488 nm).

![Figure 2. Structures of the fluorescent dyes (123, 124, 126 and 128) and corresponding succinimidyl esters (125, 127 and 129) which were used for the preparation of the fluorescent dibenzodiazepinone-type MR ligands.](image-url)
Treatment of the DIBA-derived primary amine precursor 10 and 96 (synthesis presented in chapter 3) with the pyrylium dye Py-5 (123) (cf. Figure 2) gave the fluorescent ligands 130 and 133 (cf. Scheme 2). Likewise, treatment of 10 and 96 with the succinimidyl esters 125, 127 or 129 (cf. Figure 2), resulted in the fluorescently labeled DIBA derivatives 131-136 (cf. Scheme 2).

Scheme 1. Conversion of pyrylium to pyridinium entities through reaction with primary amines.

Scheme 2. Synthesis of the fluorescently labeled MR ligands 130-136. Reagents and conditions: (a) triethylamine, DMF, rt, 2 h, 21% for 130, 34% for 133; (b) DIPEA, DMF, rt, 1-2 h, 28-41% for 131, 132 and 134-136.

4.2.2. Stability of the fluorescent ligand 136

The fluorescent M2R ligand 136 was investigated with respect to its stability under assay-like
conditions (PBS pH 7.4, 22 °C). No decomposition was observed within the incubation period of 48 h (cf. Figure 3).

![Figure 3](image)

**Figure 3.** HPLC analysis of 136 after incubation in PBS (pH 7.4) at 23 °C for up to 48 h. 136 showed no decomposition. HPLC conditions see experimental section.

### 4.2.3. Muscarinic receptor affinity and selectivity

The dibenzodiazepinone-type amine precursors 10 and 96, and the fluorescent ligands 130-136 were investigated in equilibrium competition binding experiments using the orthosteric antagonist radioligand \[^{3}H\]N-methylscopolamine (\[^{3}H\]NMS, \[^{3}H\]6) and live CHO cells stably expressing the human MR subtypes M₁-M₅. The results, expressed as Kᵢ values, are listed in Table 1. Figure 4A shows the sigmoidal curves of fluorescent ligands 130-136 at M₂R. The fluorescent ligands derived from amine precursor 96 (133-136), containing the basic piperazine moiety, exhibited higher M₂R affinities compared to the compounds derived from amine precursor 10 (130-132) (cf. Table 1), which is reflected by the affinities of the precursors 10 and 96 (Kᵢ = 14 and 0.22 nM, respectively). Compound 136, which bears two sulfonic acid groups at the fluorophore, proved to be the fluorescent ligand with the highest M₂R affinity (Kᵢ = 0.76 nM), suggesting that a negative net charge at the fluorophore is advantageous for M₂R binding. The \[^{3}H\]NMS displacement curves of ligand 136 at intact CHO-hM₂ cells (x = 1-5) are shown in Figure 4B. Obviously, the bulky fluorophore in 136 doesn’t prevent receptor binding of the dibenzodiazepinone pharmacophore. Whereas the 96-derived fluorescent ligands 133-136 showed a preference for the M₂ receptor, the fluorescent ligands derived from 10 exhibited no M₂ over M₄ receptor preference (cf. Table 1). For all compounds the M₂R selectivity was most pronounced toward the M₂R and the M₅R. Interestingly, compared to the amine precursor 96, the M₂R preference of all fluorescent ligands was less pronounced (cf. Table 1), indicating that a putative dualsteric binding mode of these ligands didn’t result in increased M₂ selectivity.
Figure 4. (A) Concentration-dependent effects of compounds 130-136 on $[^{3}H]$NMS (c = 0.2 nM) equilibrium binding at intact CHO-hM2 cells. (B) Concentration-dependent effects of compound 136 on equilibrium binding of $[^{3}H]$NMS at intact CHO-hMx cells (x = 1-5) (concentration of $[^{3}H]$NMS: 0.2 nM (M1R-M3R), 0.1 nM (M4R), 0.3 nM (M5R)). Data were analyzed by four-parameter logistic fits. Data represent mean values ± SEM from at least three independent experiments (performed in triplicate).

Table 1. MR affinities ($K_{i}$ values) of the amine precursors 10 and 96, and the fluorescent ligands 130-136 obtained from equilibrium competition binding studies with $[^{3}H]$NMS at live CHO-hMx cells (x = 1-5).

<table>
<thead>
<tr>
<th>Comp.</th>
<th>dye&lt;sup&gt;a&lt;/sup&gt;</th>
<th>$K_{i}$ [nM]</th>
<th>slope&lt;sup&gt;b&lt;/sup&gt;</th>
<th>$K_{i}$ [nM]</th>
<th>slope&lt;sup&gt;b&lt;/sup&gt;</th>
<th>$K_{i}$ [nM]</th>
<th>slope&lt;sup&gt;b&lt;/sup&gt;</th>
<th>$K_{i}$ [nM]</th>
<th>slope&lt;sup&gt;b&lt;/sup&gt;</th>
<th>$K_{i}$ [nM]</th>
<th>slope&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>-</td>
<td>n.d.</td>
<td>14 ± 3.2</td>
<td>-0.98 ± 0.07</td>
<td>n.d.</td>
<td>15 ± 3.2</td>
<td>-0.83 ± 0.07</td>
<td>230 ± 1.1</td>
<td>n.d.</td>
<td>14 ± 3.2</td>
<td>-0.83 ± 0.07</td>
</tr>
<tr>
<td>96</td>
<td>-</td>
<td>7.4 ± 1.6</td>
<td>0.22 ± 0.03</td>
<td>-1.0 ± 0.14</td>
<td>190 ± 0.67</td>
<td>0.11</td>
<td>0.97 ± 0.09</td>
<td>17 ± 1.0</td>
<td>n.d.</td>
<td>14 ± 3.2</td>
<td>-0.83 ± 0.07</td>
</tr>
<tr>
<td>130</td>
<td>Py-5</td>
<td>36 ± 6.1</td>
<td>1.7 ± 3.2</td>
<td>-1.2 ± 0.20</td>
<td>86 ± 12</td>
<td>0.06</td>
<td>1.1 ± 0.04</td>
<td>30 ± 0.09</td>
<td>n.d.</td>
<td>14 ± 3.2</td>
<td>-0.83 ± 0.07</td>
</tr>
<tr>
<td>131</td>
<td>S0223</td>
<td>140 ± 22</td>
<td>54 ± 5.4</td>
<td>-1.6 ± 0.14</td>
<td>640 ± 38</td>
<td>0.11</td>
<td>3.2 ± 0.09</td>
<td>63 ± 0.16</td>
<td>n.d.</td>
<td>14 ± 3.2</td>
<td>-0.83 ± 0.07</td>
</tr>
<tr>
<td>132</td>
<td>S0436</td>
<td>400 ± 37</td>
<td>150 ± 24</td>
<td>-1.5 ± 0.25</td>
<td>960 ± 230</td>
<td>0.11</td>
<td>9.6 ± 0.22</td>
<td>240 ± 0.24</td>
<td>n.d.</td>
<td>14 ± 3.2</td>
<td>-0.83 ± 0.07</td>
</tr>
<tr>
<td>133</td>
<td>Py-5</td>
<td>16 ± 1.6</td>
<td>3.1 ± 0.41</td>
<td>-1.9 ± 0.23</td>
<td>150 ± 9.9</td>
<td>0.02</td>
<td>4.6 ± 0.18</td>
<td>71 ± 0.20</td>
<td>n.d.</td>
<td>14 ± 3.2</td>
<td>-0.83 ± 0.07</td>
</tr>
<tr>
<td>134</td>
<td>S0223</td>
<td>18 ± 1.7</td>
<td>4.5 ± 0.47</td>
<td>-2.3 ± 0.16</td>
<td>88 ± 13</td>
<td>0.14</td>
<td>3.7 ± 0.09</td>
<td>7.7 ± 0.11</td>
<td>n.d.</td>
<td>14 ± 3.2</td>
<td>-0.83 ± 0.07</td>
</tr>
<tr>
<td>135</td>
<td>S0436</td>
<td>12 ± 2.7</td>
<td>1.4 ± 0.18</td>
<td>-1.3 ± 0.14</td>
<td>68 ± 2.4</td>
<td>0.03</td>
<td>2.5 ± 0.07</td>
<td>1.9 ± 0.15</td>
<td>n.d.</td>
<td>14 ± 3.2</td>
<td>-0.83 ± 0.07</td>
</tr>
<tr>
<td>136</td>
<td>S0387</td>
<td>5.9 ± 1.1</td>
<td>0.76 ± 0.12</td>
<td>-1.3 ± 0.12</td>
<td>82 ± 0.99</td>
<td>0.02</td>
<td>0.99 ± 0.12</td>
<td>0.99 ± 0.12</td>
<td>n.d.</td>
<td>14 ± 3.2</td>
<td>-0.83 ± 0.07</td>
</tr>
</tbody>
</table>

<sup>a</sup>Fluorescent dye used for the preparation of the respective fluorescent ligand. <sup>b</sup>Curve slope of the four-parameter logistic fit. Mean values ± SEM from 3-5 independent experiments (each performed in triplicate). $K_{d}$ values<sup>44</sup> / applied concentrations of $[^{3}H]$NMS: M1R: 0.12 / 0.2 nM; M2R: 0.090 / 0.2 nM; M3R: 0.089 / 0.2 nM; M4R: 0.040 / 0.1 nM; M5R: 0.24 / 0.3 nM.

4.2.4. Fluorescence properties of compounds 133-136.

The fluorescence quantum yields were determined (reference: cresyl violet perchlorate) for
the fluorescent ligands 133-136 in PBS (pH 7.4) and in PBS with 1% bovine serum albumin (BSA) to study the influence of proteins on the quantum yield (cf. Table 2). By selecting compounds 133-136 all types of fluorophores, used in this work, were covered.

**Table 2.** Fluorescence properties of the fluorescent ligands 133-136 in PBS and PBS containing 1% BSA: excitation/emission maxima and fluorescent quantum yields $\Phi$ (reference: cresyl violet perchlorate).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dye*</th>
<th>PBS $\lambda_{ex}/\lambda_{em}$</th>
<th>PBS $\Phi$ (%)</th>
<th>PBS+1% BSA $\lambda_{ex}/\lambda_{em}$</th>
<th>PBS+1% BSA $\Phi$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>133</td>
<td>Py-5</td>
<td>460/713</td>
<td>8.9</td>
<td>484/643</td>
<td>23.8</td>
</tr>
<tr>
<td>134</td>
<td>S0223</td>
<td>645/663</td>
<td>19.1</td>
<td>655/675</td>
<td>42.1</td>
</tr>
<tr>
<td>135</td>
<td>S0436</td>
<td>648/665</td>
<td>20.9</td>
<td>660/678</td>
<td>30.1</td>
</tr>
<tr>
<td>136</td>
<td>S0387</td>
<td>653/669</td>
<td>17.7</td>
<td>656/672</td>
<td>29.0</td>
</tr>
</tbody>
</table>

*Fluorescent dye used for the preparation of the respective fluorescent ligand.

All the investigated fluorescent ligands showed a higher quantum yield in PBS with 1% BSA compared to neat PBS (cf. Table 2). The increase in fluorescence quantum yield by adding BSA was most pronounced for 133 and 134 (> 2-fold). This phenomenon can be explained by hydrophobic and electrostatic interactions between the fluorophores and the protein resulting in a reduced molecular motion of the fluorophore and a changed chemical environment.

**Figure 5.** Excitation and corrected emission spectra (recorded at 22 °C) of the fluorescent ligands 133-136 dissolved in PBS supplemented with 1% BSA.

The excitation and corrected emission spectra of 133-136 in PBS containing 1% BSA are depicted in Figure 5, which demonstrates the considerable difference in Stoke’s shifts between
the Py-5 labeled ligand (133) and the cyanine dye labeled ligands (134-136), as well as the suitability of 133 to be excited with an argon laser (488 nm) and the compatibility of 134-136 with an excitation by the red diode laser (635 nm).

4.2.5. Flow cytometric M₂R binding studies with the fluorescent MR ligands 135 and 136

4.2.5.1. Saturation binding studies

Fluorescent ligands 135 and 136, which showed excellent M₂R affinity (Kᵢ values < 1.5 nM, cf. Table 1) were used for binding studies with flow cytometry. Saturation binding experiments performed with 135 and 136 at intact CHO-hM₂R cells, afforded Kᵣ values of 2.4 nM and 1.0 nM, respectively (cf. Figure 6A and 6B, Table 3), which were in good agreement with the Kᵢ values (1.4 and 0.76 nM, respectively, cf. Table 1) obtained from competition binding experiments with [³H]NMS at live CHO-hM₂R cells. At concentrations corresponding to the Kᵣ value, unspecific binding amounted to around 10% of total binding for both fluorescent ligands (cf. Figure 6A and 6B). The orthosteric antagonist atropine (7), used to determine unspecific binding, was capable of completely preventing one-site (monophasic) specific binding of the fluorescent ligands, indicating that 135 and 136 bind to the orthosteric binding pocket of the M₂R.

In addition, saturation binding experiments were performed with 136 at intact CHO-hM₁R cells as well as at intact CHO-hM₄R cells (cf. Figure 6C and 6D), resulting in Kᵣ values of 6.5 nM and 8.9 nM, respectively, which were in good agreement with the Kᵢ values (M₁R: 5.9 nM, M₄R: 2.5 nM) obtained from competition binding experiments with [³H]NMS at live CHO-hM₁R and CHO-hM₄R cells (cf. Table 1).
Figure 6. Representative saturation isotherms (specific binding, dashed line) obtained from flow cytometric saturation binding experiments performed with 135 (A) and 136 (B) at intact CHO-hM2 cells as well as with 136 at intact CHO-hM1 cells (C) and intact CHO-hM4 cells (D). Unspecific binding was determined in the presence of atropine (500-fold excess). Cells were incubated with the fluorescent ligands at 22 °C in the dark for 2 h. Experiments were performed in duplicate. Measurements were performed with a FACSCalibur flow cytometer (Becton Dickinson). Specific binding data were analyzed by an equation describing one-site (monophasic) binding. Error bars of specific binding represent propagated errors calculated according to the Gaussian law of errors. Error bars of total and unspecific binding represent the mean ± SEM from at least two independent experiments (each performed in duplicate).

The association and dissociation kinetics of 136 was determined at intact CHO-hM2R cells at 22 °C using flow cytometry. The association curve reached a plateau after approx. 120 min (cf. Figure 7A). The dissociation of 136 from the M2R was slow ($t_{1/2} = 52$ min) and incomplete, reaching a plateau at 83% of initial specific binding of 136 (cf. Figure 7B). However, the kinetically derived dissociation constant $K_d(\text{kin})$, calculated according to $K_d(\text{kin}) = k_{\text{off}}/k_{\text{on}}$, amounted to 2.4 nM and was in good agreement with the $K_d$ value (1.0 nM) obtained from saturation binding experiments. An overview of the M2R binding characteristics of ligand 136, determined by flow cytometric binding studies, is provided in Table 3.
4.2.5.2. Competition binding.

The suitability of fluorescent ligand 136 as reference compound for the determination of M2R ligand affinities was explored in competition binding experiments. The fluorescent ligand 136 was used at a concentration corresponding to its \( K_d \) value (1 nM). Selected standard MR agonists (2), antagonists (7, 8, 11) and allosteric modulators (14, 15, 16) were investigated by equilibrium competition binding at live CHO-hM2R cells using flow cytometry. All types of MR ligands (orthosteric (2, 7), dualsteric (8, 11) and allosteric (14, 15 and 16)) were capable of completely inhibiting specific binding of 136, resulting in sigmoidal curves that reached 0% specific binding of 136 (cf. Figure 8A and 8B). These results were indicative of a competitive mechanism between 136 and the investigated MR ligands. The apparent \( K_i \) values (shown in Table 4) were consistent with the \( K_i \) or IC\(_{50}\) values obtained from equilibrium binding studies with \([^3H]NMS\) in the presence of the MR ligands 2, 7, 8, 11 and 14-16.

Table 3. M2R binding data of the fluorescent ligand 136 determined using flow cytometry.

<table>
<thead>
<tr>
<th>Saturation binding</th>
<th>Binding kinetics</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_d) (sat) [nM](^a)</td>
<td>( K_d) (kin) [nM](^b)</td>
</tr>
<tr>
<td>1.0 ± 0.2</td>
<td>2.4 ± 0.22</td>
</tr>
</tbody>
</table>

\(^a\)Dissociation constant determined by saturation binding at live CHO-hM2R cells; mean ± SEM from three independent experiments (performed in duplicate). \(^b\)Kinetically derived dissociation constant ± propagated error \((K_d(\text{kin}) = k_{off}/k_{on})\). \(^c\)Association rate constant ± propagated error, calculated from \(k_{obs}, k_{off}\) and the applied fluorescent ligand concentration (cf. experimental section). \(^d\)Dissociation rate constant and half-life; mean ± SEM from two independent experiments (performed in duplicate).
Figure 8. Concentration-dependent effects of various reported orthosteric (2, 7) and dualsteric (8, 11) (A), as well as allostERIC (14, 15, 16) (B) MR ligands on M_2R equilibrium binding of 136 (c = 1 nM) determined at intact CHO-hM_2 cells using a FACSCalibur flow cytometer.

Table 4. M_2R binding data (K_i or IC_{50} values) of various orthosteric (2, 7), allostERIC (14, 15, 16) and dualsteric (8, 11) MR ligands determined with 136 or [^3H]NMS.

<table>
<thead>
<tr>
<th>Compound</th>
<th>136</th>
<th>[^3H]NMS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_i</td>
<td>K_i or IC_{50}**</td>
</tr>
<tr>
<td>2</td>
<td>210 ± 5.3</td>
<td>210 ± 59^{ab}</td>
</tr>
<tr>
<td>7</td>
<td>1.9 ± 0.55</td>
<td>0.94 ± 0.19^{ab}</td>
</tr>
<tr>
<td>8</td>
<td>6.2 ± 2.4</td>
<td>2.0 ± 0.2^{ab}</td>
</tr>
<tr>
<td>11</td>
<td>2.1 ± 0.27</td>
<td>0.79 ± 0.10^{ab}</td>
</tr>
<tr>
<td>14</td>
<td>2700 ± 450</td>
<td>2200 ± 410^{**c}</td>
</tr>
<tr>
<td>15</td>
<td>250 ± 44</td>
<td>460 ± 130^{**c}</td>
</tr>
<tr>
<td>16</td>
<td>6900 ± 1200</td>
<td>&gt;10000^{**c}</td>
</tr>
</tbody>
</table>

^a^Determined by flow cytometric equilibrium binding studies with 136 (c = 1 nM) in the presence of increasing concentrations of the respective MR ligand at live CHO-hM_2 cells; mean values ± SEM from at least two independent experiments (performed in duplicate). ^b^Determined by equilibrium competition binding with [^3H]NMS (c = 0.2 nM) at live CHO-hM_2 cells; mean ± SEM from at least three independent experiments (performed in triplicate). ^c^IC_{50} values obtained from nonlinear four-parameter logistic curve analyses of data characterizing the inhibition of [^3H]NMS (c = 0.2 nM) equilibrium binding at live CHO-hM_2 cells; mean ± SEM from at least 3 independent experiments (performeD in triplicate).

4.2.5.3. Saturation binding of the fluorescent ligand 136 in the presence of the allostERIC modulator 15.

On one hand, saturation binding experiments with 136 at the M_2R, using the orthostERIC antagonist 7 to determine unspecific binding (cf. Figure 6B), suggested an interaction of 136 with the orthostERIC site of the M_2R. On the other hand, M_2R equilibrium binding of 136 in the presence of the allostERIC modulators 14, 15 and 16 indicated a competitive mechanism between 136 and the allostERIC modulators (cf. Figure 8B). These findings are consistent with a dulasteric binding mode of ligand 136 at the M_2R. To further elucidate the competitive mechanism between ligand 136 and the allostERIC modulator W84 (15), saturation binding experiments were performed with the fluorescent ligand 136 in the presence of 15. This kind
of experiment is equivalent to the Schild analysis based on functional studies with agonists in the presence of antagonists, and was used, e.g. to prove the hypothesis of a competitive interplay between the allosteric modulator brucine and the fluorescent pirenzepine derivative Bo(22)Pz at the M1R. Figure 9 shows the saturation isotherms of binding of 136 to the M2R in the absence or presence of different concentrations (0.1, 0.3, 1 and 3 μM) of the allosteric modulator 15. 15 caused a parallel rightward shift of occupancy curves, going along with an apparent decrease in the affinity of 136. Based on the shift of the $K_d$ value a ‘Schild’ plot was constructed (cf. Figure 9). The slope factor was not significantly different from unity (slope = 0.85 ± 0.06 (n = 2), P > 0.2), supporting the hypothesis of a competitive mechanism between 15 and 136 and dualsteric binding of 136 at the M2R.

**Figure 9.** Saturation binding of 136 in the presence of increasing concentrations of 15. Presented are saturation isotherms of specific binding of 136 to the M2R in linear scale (left) and semi-logarithmic scale (middle), as well as the “Schild” regression (right) resulting from the rightward shifts (Δ$pK_d$) of the saturation isotherms (log(r-1) plotted vs. log(concentration 15), where $r = 10^{ΔpK_d}$). The presence of the allosteric modulator 15 led to a parallel rightward shift of the saturation isotherms of 136. The slope of the linear “Schild” regression was not different from unity (P > 0.2, assessed based on the slope mean value ± SEM (0.85 ± 0.06) from two sets of independent saturation binding experiments (performed in duplicate)) indicating a competitive interaction between 136 and 15. Data represent mean values ± SEM from at least two independent experiments (performed in duplicate).

### 4.2.6. Application of the fluorescent ligands 135 and 136 to high content imaging

#### 4.2.6.1. Saturation binding.

The fluorescent ligands 135 and 136 were also applied in plate reader-based, high-content imaging M2R binding assays, using live CHO-hM2R cells. The fluorescent ligands 135 and 136 were incubated with CHO-hM2R cells for 60 min at 22 °C, and directly (without washing of the cells) imaged by the ImageXpress (IX) Ultra plate reader. Figures 10A and 10B show representative saturation binding curves of 135 and 136. The $K_d$ values amounted to 13 nM and 4.8 nM, respectively. Due to the strong adsorption of the fluorescent ligands 135 and 136 to the 96 well plate (Grenier 655090), high levels of unspecific binding were detected (cf. Figure 10A and 10B). The application of a washing step (HBSS+0.1% BSA) after the incubation for 60 min at 22 °C, followed by immediate acquisition of the images, resulted in
considerably lower unspecific binding (at concentrations around the $K_d$ value ca 5% (135) and ca 2% (136) of total binding) and unaffected $K_d$ values (cf. Figure 10C and 10D). The disadvantage of the washing step is the loss of equilibrium conditions, however, as the fluorescent ligands exhibit low off rates (cf. Figure 7B), the fraction of dissociated ligand during the washing step is marginal.

The $K_d$ values obtained by high content imaging were slightly higher than the $K_i$ values (1.4 nM for 135 and 0.76 nM for 136) derived from radioligand competition binding experiments with $[^3H]$NMS at the $M_2$R, as well as compared to $K_d$ values (2.4 nM for 135 and 1.0 nM for 136) obtained from saturation binding using flow cytometry. This deviation might be caused by the strong adsorption of the fluorescent ligands to the plates used for high-content imaging, leading to a decrease in the concentration of ‘free’ fluorescent ligand. Figure 11 shows representative images acquired with the IX Ultra plate reader after incubation with the fluorescent ligand 136 ($c = 10$ nM) for 60 min followed by a washing step at $M_2$R. A clear difference between total and unspecific binding of 136 was observed. $K_i$ and $K_d$ values of 135 and 136 are summarized in Table 5.

**Figure 10.** Representative saturation isotherms (specific binding, dashed line) of 135 and 136 obtained from high-content imaging saturation binding experiments at intact CHO-hM2 cells. (A) (B) Cells were incubated with the fluorescent ligand at 22 °C in the dark for 1 h and directly imaged. Unspecific binding was determined in the presence of atropine (1 $\mu$M). (C) (D) Cells were incubated with the fluorescent ligand at 22 °C in the dark for 1 h, plates were washed with HBSS+0.1% BSA before acquisition of the images. Unspecific binding was determined in the presence of atropine (500-fold excess). Experiments were performed in triplicate. Measurements were performed with an IX Ultra Confocal Plate Reader (Molecular Devices). Specific binding data were analyzed by an equation describing one-site (monophasic) binding. Error bars of specific binding represent propagated errors calculated according to the Gaussian law of errors. Error bars of total and unspecific binding represent the SEM (n = 3).
Figure 11. Binding of the fluorescent ligand 136 (c = 10 nM) to live CHO-hM2 cells investigated by high-content imaging. Images (400 x 400 pixels from original 1000 x 1000 acquisition) were acquired with an IX Ultra platereader after 60 min of incubation in the dark at 22 °C from one of saturation binding experiments performed with 136. The cells were washed with HBSS+0.1% BSA before imaging. Unspecific binding of 136 was determined in the presence of 500-fold excess of atropine (lower panel). Presented are Hoechst-33342-stained nuclei in greyscale (left), the fluorescence detected in the Cy5 channel in greyscale (centre), and the overlay (right).

Table 5. Comparison of M2R binding data of 135 and 136.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>135</td>
<td>1.4 ± 0.2</td>
<td>2.4 ± 0.6</td>
<td>13 ± 2.5</td>
<td>8.9 ± 1.2</td>
</tr>
<tr>
<td>136</td>
<td>0.76 ± 0.11</td>
<td>1.0 ± 0.2</td>
<td>4.8 ± 1.5</td>
<td>3.9 ± 0.78</td>
</tr>
</tbody>
</table>

$^a$ $K_i$ values taken from Table 1. $^b$ Dissociation constant from flow cytometric saturation binding studies at live CHO-hM2 cells; mean ± SEM from three independent experiments (performed in duplicate). $^c$ Dissociation constant from high-content imaging saturation binding studies at live CHO-hM2 cells (without performing washing step); mean ± SEM from three independent experiments (performed in triplicate). $^d$ Dissociation constant from high-content imaging saturation binding studies at live CHO-hM2 cells (washing step was applied); mean ± SEM from three independent experiments (performed in triplicate).

4.2.6.2. Competition binding.

Ligands 135 and 136 were used as fluorescent probes to study a range of known muscarinic receptor ligands (including orthosteric antagonist 6 and 7, orthosteric agonist 2 and 3, allosteric modulators 14, 15 and 16). CHO-hM2 cells were grown in 96-well plates and incubated with 135 or 136 (fixed concentration, 10 nM) and 2, 3, 6, 7, 14, 15 or 16 at increasing concentrations at room temperature for 1 h. Images were directly acquired with the
ImageXpress (IX) Ultra plate reader after incubation without washing. Representative images, obtained from competition binding of 136 with 2, 6 or 7 are depicted in Figure 12A. Competition binding curves were generated using the mean of arbitrary intensity units from each well (cf. Figure 12B and 12C), and $K_i$ values were calculated using the Cheng-Prusoff equation, using the $K_d$ values (8.9 nM for 135 and 3.9 nM for 136) determined in high-content imaging saturation binding studies (cf. Table 5). All the orthosteric ligands 2, 3, 6 or 7 investigated were capable of totally inhibiting (displacing) specific M$_2$R binding of ligands 135 and 136, which suggested a competitive-like mode between the studied compounds and the fluorescent labeled ligands at the orthosteric receptor site. Moreover, the allosteric modulators 14, 15 and 16 also fully displaced 136 from the M$_2$ receptor. In the case of using 136 as the fluorescent probe, the $K_i$ values of 2, 7, 14, 15 and 16 (cf. Table 6) correlated well with the $K_i$ values obtained from flow cytometric competition binding studies (cf. Table 4). However, when using 135 as fluorescent probe, the $K_i$ values obtained for 2, 6 and 7 were slightly higher (ca 3-fold) than the $K_i$ values obtained from competition binding experiments with 136. The binding constants ($K_i$ values) of several MR ligands obtained in these experiments are shown in Table 6.

![Image](image.png)

**Figure 12.** (A) Representative thumbnail fluorescence images acquired with an IX Ultra plate reader of a high-content imaging competition binding assay performed with the fluorescent ligand 136 (c = 10 nM) and 2, 6 or 7 at live CHO-hM$_2$ cells in a 96-well plate. Each image from one well represents an area 400 x 400 μm. (B) Concentration-dependent effects of the orthosteric MR ligands 2, 6 or 7 on M$_2$R equilibrium binding of 135 determined at intact CHO-hM$_2$ cells using high-content imaging. (C) Concentration-dependent effects of various reported orthosteric (2, 3, 6 or 7), allosteric (14-16) MR
ligands on equilibrium binding of 136 determined at intact CHO-hM2 cells using high-content imaging.

Table 6. Comparison of M2R binding data (K_i or IC_{50} values) of various orthosteric (2, 3, 6 and 7), allosteric (14-16) MR ligands determined with 135, 136 or [3H]NMS.

<table>
<thead>
<tr>
<th>Compound</th>
<th>135 K_i [nM]</th>
<th>136 K_i [nM]</th>
<th>[3H]NMS K_i or IC_{50} [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>950 ± 220</td>
<td>360 ± 26</td>
<td>210 ± 59^b</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>800 ± 330</td>
<td>9300^c</td>
</tr>
<tr>
<td>6</td>
<td>2.6 ± 1.4</td>
<td>0.69 ± 0.14</td>
<td>0.2^d</td>
</tr>
<tr>
<td>7</td>
<td>15 ± 4.8</td>
<td>4.8 ± 0.92</td>
<td>0.94 ± 0.19^b</td>
</tr>
<tr>
<td>14</td>
<td>-</td>
<td>3900 ± 760</td>
<td>2200 ± 410**</td>
</tr>
<tr>
<td>15</td>
<td>-</td>
<td>1000 ± 130</td>
<td>460 ± 130**</td>
</tr>
<tr>
<td>16</td>
<td>-</td>
<td>5000 ± 1000</td>
<td>&gt;10000**</td>
</tr>
</tbody>
</table>

^a Determined by high-content imaging equilibrium binding studies with 135 (c = 10 nM) or 136 (c = 10 nM) in the presence of increasing concentrations of the respective MR ligand at live CHO-hM2 cells; mean ± SEM from three independent experiments (performed in duplicate). ^b K_i values taken from Table 4. ^c Jakubík et al.45. ^d Dei et al.46. ^e IC_{50} values obtained from nonlinear four-parameter logistic curve analyses of data characterizing the inhibition of [3H]NMS (c = 0.2 nM) equilibrium binding at live CHO-hM2 cells; mean ± SEM from at least three independent experiments (performed in triplicate).

4.2.6.3. Study of the effect of the allosteric modulator 15 on saturation binding of fluorescent ligand 136.

A series of saturation binding experiments to investigate the effect of the allosteric modulator 15 on the M2R saturation binding properties of fluorescent ligand 136 was also performed using high content imaging. Figure 13 shows the curves of saturation binding of 136 in the absence or presence of varied concentrations (0.3, 1, 2 and 3 μM) of 15 performed at CHO-hM2 cells. The results were comparable with the results obtained from the same type of experiment performed using flow cytometry. The presence of 15 led to a parallel rightward shift of the saturation isotherms of ligand 136. The slope of the linear ‘Schild’ regression was not different from unity (slope = 0.93 ± 0.11 (n = 3), P > 0.5), suggesting again a competitive interaction between ligand 136 and 15. The ‘pA_2’ value of 15 derived from the ‘Schild’ regression (‘pA_2’ = 6.80, Figure 13) was in good agreement with the ‘pA_2’ value obtained from the flow cytometry-based ‘Schild’ analysis (‘pA_2’ = 7.27, Figure 9).

Figure 13. Saturation binding of 136 in the presence of increasing concentrations of 15. Presented are saturation isotherms of specific binding of 136 to the M2R in linear scale (left) and semi-logarithmic scale (right).
scale (middle), as well as the “Schild” regressions (right) resulting from the rightward shifts ($\Delta pK_d$) of the saturation isotherms ($\log(r-1)$ plotted vs. $\log($concentration $15$), where $r = 10^{\Delta pK_d}$). The presence of the allosteric modulator $15$ led to a parallel rightward shift of the saturation isotherms of $136$. The slope of the linear “Schild” regression was not different from unity ($P > 0.5$, assessed based on the slope mean value ± SEM ($0.93 ± 0.11$) from three sets of independent saturation binding experiments) indicating a competitive interaction between $136$ and $15$. Data represent mean values ± SEM from three independent experiments (performed in triplicate).

4.2.7. Application of the fluorescent ligand $136$ to confocal microscopy

The fluorescent ligand $136$ was also applied in confocal microscopy using live CHO-hM$_2$R cells. As shown in Figure 14, a clear difference between the total and unspecific binding of $136$ (30 nM) to CHO-hM$_2$R cells was detected by confocal microscopy after different incubation times (5-45 min). Unspecific binding of $136$ was determined in the presence of the MR antagonist atropine (10 μM; Figure 14A vs. 14B). The major fraction of fluorescence appeared to be associated to the cell membrane. An increase in intracellular fluorescence was not observed over time.
Figure 14. Binding of the fluorescent ligand 136 to CHO-hM2R cells at 30 °C, visualized by confocal microscopy after 5, 15, 25, 35 and 45 min. (A) Total binding of 136 (30 nM). (B) Unspecific binding of 136 (30 nM) determined in the presence of atropine (10 μM). Images were acquired with a Zeiss LSM710 confocal microscope.

4.3. Conclusion

This work represents the first report on fluorescently labeled dibenzodiazepinone-type M2 subtype-preferring MR ligands. The dibenzodiazepinone scaffold was linked to fluorophores via two different linkers with respect to lengths and chemical nature (non-basic vs. basic), yielding seven fluorescent ligands. Six of these fluorescent probes exhibited high M2R affinity (K < 55 nM). Variations of the chemical structure of the fluorophores had less impact on M2R affinity than the type of the linker. The attachment of the cyanine dyes S0436 and S0387 through a linker containing a piperazine moiety yielded the fluorescent ligands with the highest affinity (135 and 136: K ≈ 1 nM) at M2R. Application of 135 and 136 to flow cytometry and high content imaging proved that these new fluorescent probes are suited for such techniques. The fluorescent ligand 136 was identified as a valuable, non-radiolabeled pharmacological tool for the determination of MR affinities of MR ligands. M2R binding studies with 136 in the presence of allosteric modulators strongly suggested that 136 and structurally related ligands bind simultaneously to both the orthosteric (through the dibenzodiazepinone scaffold) and the ‘common’ allosteric binding site (most likely through the fluorophores) of the M2R (dualsteric
binding mode). Owing to the low M₂R selectivity, the fluorescent ligand 136 represents a potential fluorescent probe for binding studies at the M₁R and M₄R as well, which was supported by saturation binding studies at the M₁R and M₄R revealing Kᵰ values of 6.5 and 8.9 nM, respectively. Moreover, this work suggests that anchoring an allosteric moiety (here mimicked by the fluorophore) through a linker to the dibenzodiazepinone scaffold might be a promising perspective to develop selective M₂R antagonists according to the dualsteric ligand approach.

4.4. Experimental section

4.4.1. General experimental conditions

Acetonitrile for HPLC (gradient grade) was obtained from Merck (Darmstadt, Germany). Millipore water was used throughout for the preparation of stock solutions, buffers and HPLC eluents. Bovine serum albumin (BSA) was from Serva (Heidelberg, Germany). DMF, trimethylamine and trifluoroacetic acid were obtained from (Sigma-Aldrich (Deisenhofen, Germany), and N,N-diisopropylethylamine (DIPEA) (99%) was purchased from ABCR (Karlsruhe, Germany). The pyrylium dye Py-5 was synthesized by Mengya Chen in our research group as part of her master thesis. The succinimidyl esters of the fluorescent dyes S0223 (equates to S2197), S0536 (S0436-NHS) and S0586 (S0387-NHS) were obtained from FEW Chemicals (Bitterfeld-Wolfen, Germany). [³H]N-methylscopolamine ([³H]NMS) (specific activity = 80 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO) via Hartman Analytics GmbH (Braunschweig, Germany). Atropine, N-methylscopolamine (NMS), W84 and gallamine were purchased from Sigma-Aldrich (Deisenhofen, Germany). Oxotremorine sesquifumarate was from MP Biomedicals (Eschwege, Germany). LY2119620 was from Absource Diagnostic (Munich, Germany) and AF-DX 384 was purchased from Abcam (Cambridge, UK). Xanomeline (2) was prepared according to described procedures (purity = 97%). Compound 11 was prepared in our lab according to a reported protocol. Polypropylene reaction vessels (1.5 mL) with screw cap (Süd-Laborbedarf, Gauting, Germany) were used for the synthesis of fluorescent ligands, for the investigation of chemical stabilities and for the preparation and storage of stock solutions. ¹H-NMR spectra were recorded on a Bruker Avance 600 (¹H: 600 MHz) (Bruker, Karlsruhe, Germany) with TMS as external standard. High-resolution mass spectrometry (HRMS) analysis was performed on an Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS system (Agilent Technologies, Santa Clara, CA) using an ESI source. Preparative HPLC was performed on a system from Knauer (Berlin, Germany) consisting of two K-1800 pumps and a K-2001 detector. A Kinetex-XB C18, 5 μm, 250 mm × 21 mm (Phenomenex, Aschaffenburg, Germany) served as stationary phase at a
flow-rate of 15 mL/min using mixtures of acetonitrile and 0.1% aq TFA as mobile phase. A

detection wavelength of 220 nm was used throughout. Lyophilisation of the collected fractions

was performed with an Alpha 2-4 LD apparatus (Martin Christ, Osterode am Harz, Germany)

equipped with a RZ 6 rotary vane vacuum pump (Vacuubrand, Wertheim, Germany). Analytical

HPLC analysis was performed on a system from Merck-Hitachi (Hitachi, Düsseldorf, Germany)

composed of a L-6200-A pump, an AS-2000A autosampler, a L-4000A UV detector, a D-6000

interface. A Kinetex-XB C18, 5 μm, 250 mm × 4.6 mm (Phenomenex, Aschaffenburg, Germany)

was used as stationary phase at a flow rate of 0.8 mL/min. Mixtures of acetonitrile (A) and 0.1%

aq TFA (B) were used as mobile phase (degassed by Helium purging). The following linear

gradient was applied: 0-30 min: A/B 5:95-85:15, 30-32 min: 85:15-95:5, 32-40 min: 95:5.

Detection was performed at 220 nm throughout. The oven temperature was 30 °C. The stock

solutions (concentrations: 1, 5 or 10 mM) of fluorescent ligands were prepared in DMSO/H2O

(1:1 v/v) and were stored at -80 oC.

The fluorescent ligands were characterized by 1H-NMR spectroscopy, HRMS, and RP-HPLC

analysis. The purity (RP-HPLC, detection at 220 nm) of the fluorescent ligands amounted

to >96%. Annotation concerning the 1H-NMR spectra of the fluorescent ligands (130-136): due
to a slow rotation about the exocyclic amide group on the NMR time scale, two isomers (ratios

provided in the experimental protocols) were evident in the 1H-NMR spectra.

4.4.2. Chemistry: experimental protocols and analytical data

4-((1E,3E)-4-(4-(Dimethylamino)phenyl)buta-1,3-dien-1-yl)-2,6-dimethyl-1-(4-(1-(2-oxo-

2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-

yl)butyl)pyridin-1-ium hydrotrifluoroacetate trifluoroacetate (130)

The reaction was carried out in a 1.5-mL eppendorf reaction vessel equipped with a micro stir

bar. Compound 10 (2.0 mg, 4.92 μmol) and triethylamine (4.98 mg, 6.8 μL, 49.2 μmol) were
dissolved in anhydrous DMF (300 μL) followed by the addition of Py-5 × 1 BF4" (123) (5.4 mg,
14.8 μmol) in anhydrous DMF (120 μL) and stirring at room temperature in the dark for 2 h.
10% aq TFA (corresponding to 49.2 μmol of TFA) were added. Purification of the product by
preparative HPLC (column: Kinetex XB-C18 5 μm 250 × 21 mm; gradient: 0-30 min: MeCN/0.1%
aq TFA 5:95-62:38, tR = 22 min). afforded 130 as a red solid (0.91 mg, 21%). Ratio of
configurational isomers evident in the NMR spectra: ca 1.5:1. 1H-NMR (600 MHz, MeOH-d4):
δ (ppm) 1.38-1.44 (m, 2H), 1.46-1.61 (m, 4H), 1.79-1.85 (m, 2H), 1.92-2.06 (m, 2H), 2.78 (s,
6H), 2.88-2.99 (m, 1H), 3.02 (s, 6H), 3.18-3.19 (m, 1H), 3.40-3.42 (m, 1H), 3.42-3.52 (m, 1H),
3.68-3.85 (m, 2H), 4.33-4.37 (m, 2H), 4.40 (d, J 18 Hz, 0.6H), 4.43 (d, J 18 Hz, 0.4H), 6.57 (d,
J 18 Hz, 1H), 6.76 (d, J 12 Hz, 2H), 6.92-7.01 (m, 2H), 7.25-7.40 (m, 3H), 7.43 (d, J 12 Hz,
2-((1E,3E)-5-((E)-3,3-Dimethyl-1-(6-oxo-6-((4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)amino)hexyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3H-indol-1-ium hydrotrifluoroacetate trifluoroacetate (131)

Compound 131 was prepared from 10 (2.2 mg, 5.42 μmol) and S2197 (2.4 mg, 3.63 μmol) according to the procedure for the synthesis of 130, but DIPEA (4.69 mg, 6.3 μL, 36.3 μmol) was used instead of triethylamine. Purification by preparative HPLC (column: Kinetex XB-C18 5 μm 250 × 21 mm; gradient: 0-30 min: MeCN/0.1% aq TFA 5:95-90.5:9.5, tₘᵣ = 22 min) afforded the product as a blue solid (1.62 mg, 40%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ¹H-NMR (600 MHz, MeOH-d₄): δ (ppm) 1.25-1.35 (m, 5H), 1.42-1.47 (m, 4H), 1.47-1.52 (m, 1H), 1.63-1.69 (m, 2H), 1.69 (s, 12H), 1.78-1.84 (m, 2H), 1.85-1.98 (m, 2H), 2.17 (t, J 6.0 Hz, 2H), 2.84-2.93 (m, 1H), 2.98-3.04 (m, 1H), 3.11 (t, J 6.0 Hz, 2H), 3.18-3.19 (m, 0.5H), 3.31-3.33 (m, 1H), 3.41-3.42 (m, 1H), 3.42-3.44 (m, 0.5H), 3.61 (s, 3H), 3.68-3.74 (m, 1H), 4.08 (t, J 12 Hz, 2H), 4.37 (d, J 18 Hz, 0.6H), 4.41 (d, J 18 Hz, 0.4H), 6.26 (d, J 12 Hz, 2H), 6.60 (t, J 12 Hz, 1H), 7.24-7.35 (m, 6H), 7.36-7.53 (m, 7H), 7.60-7.64 (m, 1H), 7.67-7.76 (m, 1H), 7.89-7.90 (m, 0.6H), 7.95-7.97 (m, 0.4H), 8.21-8.26 (m, 2H). HRMS (ESI): m/z [M⁺] calcd. for [C₅₆H₇₇N₆O₃⁺]: 871.5269, found: 871.5265. C₅₆H₇₇N₆O₃⁺ · C₄H₅O₄ (872.19 + 227.04).

4-((E)-3,3-Dimethyl-1-(6-oxo-6-((4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)amino)hexyl)indolin-2-ylidene)penta-1,3-dien-1-yl)-3,3-dimethyl-3H-indol-1-ium hydrotrifluoroacetate (132)

Compound 132 was prepared from 10 (1.9 mg, 4.67 μmol) and S0536 (2.2 mg, 3.14 μmol) according to the procedure for the synthesis of 130, but DIPEA (4.05 mg, 5.5 μL, 31.3 μmol) was used instead of triethylamine. Purification by preparative HPLC (column: Kinetex 5μ-XB-C18 250 × 21 mm; gradient: 0-30 min: MeCN/0.1% aq TFA 5:95-90.5:9.5, tₘᵣ = 19 min) afforded the product as a blue solid (1.43 mg, 37%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. ¹H-NMR (600 MHz, MeOH-d₄): δ (ppm) 1.26-1.35 (m, 5H), 1.43-1.47 (m, 4H), 1.48-1.54 (m, 2H), 1.64-1.69 (m, 2H), 1.71 (s, 6H), 1.72 (s, 6H), 1.77-1.84 (m, 2H), 1.84-1.89 (m, 1H), 1.92-1.99 (m, 4H), 2.18 (t, J 6.0 Hz, 2H), 2.88-2.95 (m, 2H), 3.02-3.06 (m, 1H), 3.13 (t, J 6.0 Hz, 2H), 3.18-3.19 (m, 0.5H), 3.31-3.32 (m, 1H), 3.41-3.42 (m, 0.5H), 3.43-3.46 (m, 1H), 3.66-3.80 (m, 2H), 4.08 (t, J 6.0 Hz, 2H), 4.10-4.16 (m, 2H), 4.37 (d, J 18 Hz, 0.4H), 4.41 (d, J 18 Hz, 0.4H), 6.26 (d, J 12 Hz, 2H), 6.60 (t, J 12 Hz, 1H), 7.24-7.35 (m, 6H), 7.36-7.53 (m, 7H), 7.60-7.64 (m, 1H), 7.67-7.76 (m, 1H), 7.89-7.90 (m, 0.6H), 7.95-7.97 (m, 0.4H), 8.21-8.26 (m, 2H). HRMS (ESI): m/z [M⁺] calcd. for [C₅₆H₇₇N₆O₃⁺]: 871.5269, found: 871.5265. C₅₆H₇₇N₆O₃⁺ · C₄H₅O₄ (872.19 + 227.04).
0.6H), 4.43 (d, J 18 Hz, 0.4H), 6.25 (d, J 18 Hz, 1H), 6.33-6.36 (m, 1H), 6.60-6.66 (m, 1H), 7.19-7.27 (m, 4H), 7.31-7.35 (m, 2H), 7.36-7.41 (m, 2H), 7.41-7.54 (m, 5H), 7.59-7.65 (m, 1H), 7.66-7.75 (m, 1H), 7.88-7.89 (m, 0.6H), 7.94-7.96 (m, 0.4H), 8.19-8.24 (m, 2H). RP-HPLC (220 nm): 96% (tR = 24.2 min, k = 7.4). HRMS (ESI): m/z [M+H]+ calcd. for [C59H72N5O6S]+: 993.5307, found: 993.5317. C59H72N5O6S · C4H2F3O4 (993.32 + 228.05).

4-((1E,3E)-4-(4-(Dimethylamino)phenyl)buta-1,3-dien-1-yl)-2,6-dimethyl-1-(2-(4-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)pyridin-1-ium tris(hydrotrifluoroacetate) trifluoroacetate (133)

Compound 133 was prepared from 96 (3.0 mg, 3.08 μmol), Py-5 × 1BF4 (3.65 mg, 9.90 μmol) and triethylamine (5.36 mg, 74.μL, 52.9 μmol) according to the procedure for the synthesis of 130. Purification of the product by preparative HPLC (column: Kinetex XB-C18 5 μm 250 × 21 mm; gradient: 0-30 min: MeCN/0.1% aq TFA 20:80-64:36, tR = 12 min) afforded the product as a red solid (1.31 mg, 34%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. 1H-NMR (600 MHz, MeOH-d4): (ppm) δ 1.19-1.31 (m, 4H), 1.35-1.38 (m, 1H), 1.45-1.63 (m, 1H), 1.65-1.77 (m, 2H), 1.86-1.99 (m, 2H), 2.00-2.02 (m, 1H), 2.13-2.22 (m, 1H), 2.59 (t, J 12 Hz, 2H), 2.82 (s, 6H), 2.88-2.97 (m, 3H), 3.02 (s, 6H), 3.06-3.16 (m, 4H), 3.17-3.18 (m, 1H), 3.41-3.42 (m, 1H), 3.43-3.47 (m, 1H), 3.53-3.56 (m, 2H), 3.67-3.80 (m, 2H), 4.38 (d, J 18 Hz, 0.6H), 4.42 (d, J 18 Hz, 0.4H), 4.55 (t, J 6.0 Hz, 2H), 6.57 (d, J 18 Hz, 1H), 6.75 (d, J 6.0 Hz, 2H), 6.92-7.03 (m, 2H), 7.24-7.40 (m, 3H), 7.43 (d, J 6.0 Hz, 2H), 7.46-7.54 (m, 2H), 7.51-7.70 (m, 3H), 7.72-7.75 (m, 2H), 7.88-7.93 (m, 0.6H), 7.96-7.98 (m, 0.4H). RP-HPLC (220 nm): 98% (tR = 16.6 min, k = 4.8); HRMS (ESI): m/z [M]+ calcd. for [C49H62N4O2]+: 780.4960, found: 780.4961. C49H62N4O2 · C6H3F3O3 (781.08 + 456.09).

2-((1E,3E)-5-((E)-3,3-Dimethyl-1-(6-oxo-6-((2-(4-(1-(2-oxo-2-(11-oxo-10,11-dihydro-5H-dibenzo[b,e][1,4]diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)amino)hexyl)indolin-2-ylidine)penta-1,3-dien-1-yl)-1,3,3-trimethyl-3H-indol-1-ium tris(hydrotrifluoroacetate) trifluoroacetate (134)

Compound 134 was prepared from 96 (5.8 mg, 5.95 μmol) and S2197 (3.0 mg, 4.54 μmol) according to the procedure for the synthesis of 130, but DIPEA (5.87 mg, 7.9 μL, 45.3 μmol) was used instead of triethylamine and the incubation time period was 1 h instead of 2 h. Purification by preparative HPLC (column: Kinetex XB-C18 5 μm 250 × 21 mm; gradient: 0-30 min: MeCN/0.1% aq TFA 5:95-90.5:9.5, tR = 19 min) afforded the product as a blue solid (2.06 mg, 31%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. 1H-NMR (600 MHz, MeOH-d4): δ (ppm) 1.28-1.40 (m, 5H), 1.42-1.57 (m, 5H), 1.57-1.69 (m, 4H), 1.72 (s, 12H), 1.77-1.85 (m, 2H), 1.85-2.02 (m, 2H), 2.21 (t, J 6.0 Hz, 2H), 2.63-2.65 (m, 2H),
According to the procedure for the synthesis of Compound 3,3-Dimethyl-1-(6-oxo-6-((2-(4-(1-(10,11-dihydro-5H-dibenzo[b,e]1,3-diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)amino)hexyl)indoline (136)

Compound 136 was prepared from 96 (4.46 mg, 4.58 μmol) and S0586 (2.3 mg, 2.94 μmol) according to the procedure for the synthesis of 130, but DIPEA (4.24 mg, 5.7 μL, 32.8 μmol) was used instead of trimethylamine and the incubation time period was 1 h instead of 2 h.

Purification by preparative HPLC (column: Kinetex XB-C18 5 μm 250 × 21 mm; gradient: 0-30 min: MeCN/0.1% acq TFA 5:95-90:5:9.5; tR = 18 min) afforded the product as a blue solid (1.59 mg, 30%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. H-NMR (600 MHz, MeOH-d4): δ (ppm) 1.28-1.42 (m, 5H), 1.42-1.61 (m, 5H), 1.67-1.71 (m, 4H), 1.71 (s, 6H), 1.72 (s, 6H), 1.73-1.75 (m, 1H), 1.79-1.84 (m, 2H), 1.88-1.92 (m, 2H), 1.93-2.00 (m, 5H), 2.23 (t, J 6.0 Hz, 2H), 2.63-2.76 (m, 2H), 2.78-2.98 (m, 3H), 3.02-3.07 (m, 3H), 3.18-3.19 (m, 0.5H), 3.30-3.32 (m, 3H), 3.33-3.37 (m, 3H), 3.41-3.42 (m, 0.5H), 3.43-3.45 (m, 1H), 3.69-3.79 (m, 2H), 4.09 (t, J 6.0 Hz, 2H), 4.12-4.17 (m, 2H), 4.38 (d, J 18 Hz, 0.6H), 4.42 (d, J 18 Hz, 0.4H), 6.27 (d, J 18 Hz, 1H), 6.34 (d, J 12 Hz, 1H), 6.61-6.67 (m, 1H), 7.25-7.29 (m, 4H), 7.31-7.34 (m, 2H), 7.35-7.43 (m, 3H), 7.44-7.55 (m, 4H), 7.59-7.65 (m, 1H), 7.67-7.76 (m, 1H), 7.88-7.90 (m, 0.6H), 7.96-7.97 (m, 0.4H), 8.19-8.26 (m, 2H). RP-HPLC (220 nm): 96% (tR = 21.3 min, k = 6.4). HRMS (ESI): m/z [M+H]+ calcd. for [C65H84N6O6S]+: 1105.6307, found: 1105.6309. C65H84N6O6S · C8H4F12O6 (1105.50 + 456.09).

(E)-2-((2E,4E)-5-(3,3-Dimethyl-1-(4-sulfonatobutyl)-3H-indol-1-ium-2-yl)penta-2,4-dien-1-ylidene)-3,3-dimethyl-1-(6-oxo-6-((2-(4-(1-(10,11-dihydro-5H-dibenzo[b,e]1,3-diazepin-5-yl)ethyl)piperidin-4-yl)butyl)piperazin-1-yl)ethyl)amino)hexyl)indoline-5-sulfonate trakis(hydrotrifluoroacetate) (136)
30 min: MeCN/0.1% aq TFA 5:95-90.5:9.5, \( t_R = 15 \text{ min} \) afforded the product as a blue solid (1.45 mg, 30%). Ratio of configurational isomers evident in the NMR spectra: ca 1.5:1. \(^1\)H-NMR (600 MHz, MeOH-d\(_4\)): \( \delta \) (ppm) 1.26-1.40 (m, 4H), 1.40-1.56 (m, 4H), 1.59-1.70 (m, 3H), 1.73 (s, 6H), 1.74 (s, 6H), 1.75-1.77 (m, 3H), 1.77-1.87 (m, 2H), 1.89-2.05 (m, 6H), 2.18 (t, \( J = 6.0 \text{ Hz, 2H} \)), 2.31-2.41 (m, 2H), 2.89 (t, \( J = 6.0 \text{ Hz, 2H} \)), 2.95-3.01 (m, 2H), 3.01-3.13 (m, 3H), 3.18-3.19 (m, 1H), 3.19-3.22 (m, 1H), 3.19-3.33 (m, 4H), 3.41-3.42 (m, 1H), 3.45 (d, \( J = 18 \text{ Hz, 0.6H} \)), 4.07 (t, \( J = 6.0 \text{ Hz, 2H} \)), 4.21-4.23 (m, 2H), 4.38 (d, \( J = 18 \text{ Hz, 0.6H} \)), 4.43 (d, \( J = 18 \text{ Hz, 0.4H} \)), 6.21 (d, \( J = 12 \text{ Hz, 1H} \)), 6.50 (d, \( J = 12 \text{ Hz, 1H} \)), 6.65-6.71 (m, 1H), 7.22-7.31 (m, 2H), 7.31-7.34 (m, 2H), 7.36-7.46 (m, 3H), 7.44-7.54 (m, 3H), 7.60-7.67 (m, 1H), 7.67-7.75 (m, 1H), 7.81-7.86 (m, 2H), 7.89-7.91 (m, 0.6H), 7.96-7.97 (m, 0.4H), 8.12-8.32 (m, 2H). RP-HPLC (220 nm): 99% (\( t_R = 17.6 \text{ min, } k = 5.1 \)). HRMS (ESI): m/z [M+H]\(^+\) calcd. for \( \text{C}_{65}\text{H}_{83}\text{N}_{8}\text{O}_{9}\text{S}_{2}^- \cdot \text{C}_{8}\text{H}_{4}\text{F}_{12}\text{O}_{8} \): 1185.5875, found: 1185.5896. \( \text{C}_{65}\text{H}_{83}\text{N}_{8}\text{O}_{9}\text{S}_{2}^- \cdot \text{C}_{8}\text{H}_{4}\text{F}_{12}\text{O}_{8} \) (1184.55 + 456.09).

4.4.3. Determination of fluorescence quantum yields

The determination of the fluorescence quantum yields of \( \text{133}-\text{136} \) in PBS and PBS containing 1% BSA was performed with a Cary Eclipse spectrofluorimeter and a Cary 100 UV/VIS photometer (Varian Inc., Mulgrave, Victoria, Australia) as described previously with minor modifications\(^6\). All spectra were recorded using acryl cuvettes (10 × 10 mm, Ref. 67.755, Sarstedt, Nümbrecht, Germany). Fluorescence spectra were recorded at the slit adjustments (excitation/emission) 10/5 nm and 10/10 nm. Table 7 provides an overview of the used concentrations of the fluorescent ligands and the applied excitation wavelengths. The concentration of cresyl violet perchlorate in EtOH was 2 µM. Fluorescence spectra of cresyl violet perchlorate were recorded using an excitation wavelength of 575 nm.

<table>
<thead>
<tr>
<th>Compound</th>
<th>concentration [µM]</th>
<th>excitation wavelength [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
<td>PBS + 1% BSA</td>
</tr>
<tr>
<td>133</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>134</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>135</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>136</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

4.4.4. Investigation of the chemical stability

The stability of the fluorescent ligand \( \text{136} \) was investigated in phosphate buffered saline (pH
The incubation was started by addition of 10 μL of a 5 mM stock solution of 136 (in DMSO/H2O 1:1 v/v) to 490 μL of PBS to give a final concentration of 100 μM. After 0 h, 6 h, 24 h, 48 h, an aliquot (120 μL) was taken and added to acetonitrile/H2O/2% aq TFA (2:4:4 v/v/v) (120 μL). 100 μL of the resulting solution (pH < 4) were analyzed by analytical HPLC on a system from Merck-Hitachi (Hitachi, Düsseldorf, Germany) composed of a L-6200-A pump, an AS-2000A autosampler, a L-4000A UV detector and a D-6000 interface. A Kinetex-XB C18, 5 μm, 250 mm × 4.6 mm (Phenomenex, Aschaffenburg, Germany) served as stationary phase at a flow rate of 0.8 mL/min. The following linear gradient was applied: 0-20 min: acetonitrile/0.1% aq TFA 10:90-36:64, 20-28 min: 36:64-95:5, 28-35 min: 95:5. Detection was performed at 220 nm and the oven temperature was 30 °C.

4.4.5. [3H]NMS competition binding assay

Radioligand binding studies with [3H]NMS were performed at 22 ± 1 °C. Leibovitz L-15 medium (Gibco, Life Technologies GmbH, Darmstadt, Germany) supplemented with 1% BSA (Serva, Heidelberg, Germany) (in the following referred to as L15 medium) was used as binding buffer. The effects of the fluorescent ligands 130-136 on the equilibrium binding of [3H]NMS (equilibrium competition binding assay) were determined at intact adherent CHO-hMxR cells (x = 1-5) in white 96-well plates with clear bottom (Corning Life Sciences, Tewksbury, MA; Corning cat. no. 3610) using the protocol of previously described MR binding studies with [3H]NMS with the following modification: the total volume of L15 medium per well was 200 μL instead of 188 μL, i.e. the cells were covered with L15 medium (160 μL) followed by the addition of L15 medium (20 μL) neat or containing competitors or atropine (10-fold concentrated), and L15 medium (20 μL) containing the radioligand 10-fold concentrated. The concentration of [3H]NMS was 0.2 nM (M1, M2, M3), 0.1 nM (M4) or 0.3 nM (M5) and the incubation time was 3 h throughout.

4.4.6. Flow cytometric binding experiments

All flow cytometric binding studies were performed with a FACSCalibur™ flow cytometer (Becton Dickinson, Heidelberg, Germany), equipped with an argon laser (488 nm) and a red diode laser (635 nm), instrument settings were: FSC, E-1; SSC, 280 V; Fl-4, 700–800 V. All samples were prepared and incubated in 1.5 mL reaction vessels (Sarstedt, Nümbrecht, Germany). Samples were prepared in duplicate throughout. Fluorescence signals were recorded in channel FL-4 (excitation: 635 nm, emission: 661 ± 18 nm). Measurements were stopped after counting of 20,000 gated events (highest flow rate).
4.4.6.1. Saturation binding studies at the M₁R, M₂R and M₄R and competition binding with fluorescent ligand 136 at the M₂R

CHO-hMₓR (x = 1, 2 and 4) cells were seeded in a 175-cm² culture flask 5-6 days prior to the experiment. Cells were treated with trypsin, suspended in culture medium and centrifuged. The cell pellet was re-suspended in Leibovitz’s L15 culture medium (Gibco, Life Technologies, Darmstadt, Germany) supplemented with 1% bovine serum albumin (Serva, Heidelberg, Germany), in the following referred to as L15 medium. The cell density was adjusted 1 · 10⁶ cells/mL. For saturation binding experiments, 490 μL of the cell suspension were added to reaction vessels, 5 μL of a solution of the fluorescent ligand (100-fold concentrated) in DMSO/H₂O (1:1 v/v) and 5 μL of DMSO/H₂O (1:1 v/v), were added to determine total binding. For the determination of unspecific binding (in the presence of atropine at 500-fold access to the fluorescent ligand), 490 μL of the cell suspension were added to reaction vessels, 5 μL of a solution of the fluorescent ligand (100-fold concentrated) in DMSO/H₂O (1:1 v/v) and 5 μL of a solution of atropine (100-fold concentrated) in DMSO/H₂O (1:1 v/v) were added. Compound 135 or 136 was applied at final concentrations of 0.1-15 nM and 0.1-10 nM, respectively, for M₂R binding experiments, and 136 was applied at final concentrations of 0.15-80 nM for M₁R and M₄R binding studies. The incubation period was 2 h.

For competition binding experiments with 136 (c = 1.0 nM) at CHO-hM₂R cells, 5 μL of a solution of the fluorescent ligand (100-fold concentrated) in DMSO/H₂O (1:1 v/v) and 5 μL of a solution of atropine (100-fold concentrated) in DMSO/H₂O (1:1 v/v), to determine unspecific binding (500-fold excess of atropine to 136), or 5 μL of a solution of the compound of interest (including 2, 7, 8, 11, 14, 15 and 16) (100-fold concentrated) in DMSO/H₂O (1:1 v/v), for competition binding, were premixed in the reaction vessels followed by addition of 490 μL of the cell suspension. The incubation period was 2 h.

M₂R saturation binding experiments with 136 in the presence of various fixed concentrations of 15 were performed as described above with the following modification: vessels were prefilled with 485 μL of M₂R cell suspension. For total binding, L15 medium (5 μL), L15 medium (5 μL) containing 15 100-fold concentrated, L15 medium (5 μL) containing 136 100-fold concentrated were added. For unspecific binding, L15 medium (5 μL) containing compound 136 (100-fold concentrated), L15 medium (5 μL) containing 15 (100-fold concentrated) and L15 medium (5 μL) containing atropine 100-fold concentrated (500-fold excess to compound 136) were added.
4.4.6.2. Association and dissociation kinetics of 136 at CHO-hM2R cells

For association experiments with 136 (c = 3 nM) at CHO-hM2R cells reaction vessels were prefilled with 490 μL of the cell suspension. For total binding DMSO/H2O (1:1 v/v) (5 μL) and DMSO/H2O (1:1 v/v) (5 μL) containing 136 (0.3 μM) were added. To determine unspecific binding DMSO/H2O (1:1 v/v) (5 μL) containing atropine (30 μM) and DMSO/H2O (1:1 v/v) (5 μL) containing 136 (0.3 μM) were added to the cell suspension. The incubation was stopped after different periods of time (0-140 min) by measurement of the samples. In the case of dissociation experiments, cells were preincubated with 136 at a final concentration of 5 nM for 2 h (500 μL total volume per vessel). Unspecific binding was determined in the presence of atropine at a final concentration of 1 μM. After incubation, the cells were centrifuged at room temperature for 3.5 min, the supernatant was removed by suction and the cells were covered with L15 medium (500 μL) containing atropine (2.5 μM) followed by shaking in the dark. After different periods of time (0-150 min), the samples were subjected to measurement by flow cytometry.

4.4.7. High content imaging binding experiments

4.4.7.1. Saturation and competition binding assay

One day prior to the experiment CHO-hM2R cells were seeded at 35,000-40,000 cells per well into the central 60 wells of a black/transparent 96-well plate (Grenier 655090). The medium was removed by suction, the cells were washed with HBSS containing 0.1 % BSA (in the following referred to as HBSS-BSA) (50 μL), and covered with 80 μL of HBSS-BSA containing the permeable nuclear dye H33342 (2 μg/mL, Sigma). To determine total binding HBSS-BSA (10 μL) and HBSS-BSA (10 μL) containing the fluorescent ligand (10-fold concentrated) were added. For the determination of unspecific binding and to study the effect of a compound of interest on M2R binding of the fluorescent ligand (competition binding assay) HBSS-BSA (10 μL) containing atropine or the ‘competitor’ (10-fold concentrated) and HBSS-BSA (10 μL) containing the fluorescent ligand (10-fold concentrated) were added. After incubation at room temperature in the dark for 60 min, images were acquired with the IX Ultra confocal plate reader (Molecular Devices, Sunnyvale CA) to obtain “non-washing” saturation binding curves (cf. Figure 10A and 10B), unspecific binding was determined in the presence of atropine (1 μM). After incubation at room temperature in the dark for 60 min, the medium was removed by suction and the cells were washed with HBSS-BSA (50 μL) and covered with HBSS-BSA (50 μL) followed by immediate acquisition of the images using the IX Ultra confocal plate reader to obtain “washing step applying” saturation binding curves (cf. Figure 10C and 10D),
unspecific binding was determined in the presence of atropine (500-fold excess to the fluorescent ligand). The washing process was performed within < 2 min. For competition binding experiments, unspecific binding was determined in the presence of atropine (1 µM), plates were directly imaged after incubation for 1 h. Saturation binding experiments were performed in triplicate and competition binding assays were performed in duplicate. In case of competition binding studies the 'competitor' was added to the cells 2 min prior to the addition of fluorescent ligand. One site/well was measured with the plate reader in case of competition binding with 2, 6 and 7, and two sites/well were measured in case of saturation binding studies and competition binding experiments with 3, 14, 15 and 16. The excitation laser lines of the Ultra were 405 nm (H33342), 488 nm (FITC), 561 nm (Texas Red) and 635 nm (Cy5), the emission laser lines of the Ultra were 447/60 nm (H33342), 525/50 nm (FITC), 593/40 nm (Texas Red) and 685/40 nm (Cy5).

M2R saturation binding experiments with 136 in the presence of various fixed concentrations of 15 were performed as described above with the following modification: the cells were covered with 70 µL of HBSS-BSA instead of 80 µL to compensate the extra addition of HBSS-BSA (10 µL) containing 15 (10-fold concentrated). The washing step prior to the measurement was performed.

### 4.4.8. Confocal Microscopy

One day prior to the experiment CHO-hM2R cells were trypsinized and seeded in Nunc LabTekTM II chambered coverglasses with 8 chambers (Thermo fisher scientific) (ca. 80000 cells/well). The culture medium was removed, the cells were washed with HBSS-BSA (200 µL) and covered with HBSS-BSA (320 µL). HBSS-BSA (40 µL) and HBSS-BSA (40 µL) with the fluorescent probe (10 fold concentrated) was added for total binding. For unspecific binding HBSS-BSA (40 µL) with the competing agent atropine (final concentration: 10 µM) and HBSS-BSA (40 µL) with the fluorescent probe (10 fold concentrated) were added. Images of total and unspecific binding were acquired after an incubation period of 5-45 min. Confocal microscopy was performed with a zeiss LSM710 confocal microscope. The objective was 63x magnification with oil (1.4NA). The excitation laser lines were 405 nm (2.0%) and 633 nm (10.0%), filter settings were 410-514 nm and 638-759 nm, pinhole settings are 44 µm for both DAPI and Cy5 channels.

### 4.5. Data processing

Retention (capacity) factors $k$ were calculated from retention times ($t_R$) according to $k =$
Raw data from flow cytometric experiments were processed with the aid of FloJo software to obtain geometrical mean values of FL-4. Fluorescence images from high content imaging were analyzed using the granularity analysis (2-3-μm-diameter granules; MetaXpress 5.3, Molecular Devices) to obtain values of arbitrary intensity units. Intensity thresholds were adapted to maximize the identification of specifically bound fluorescent ligand (without distinguishing membrane from intracellular localization), by reference to total and unspecific plate controls. Specific binding data from saturation binding experiments (flow cytometry, high content imaging) were plotted against the fluorescent ligand concentration and analyzed by a two-parameter equation describing hyperbolic binding (one site-specific binding, GraphPad Prism) to obtain $K_d$ values. Unspecific binding data from saturation binding experiments were fitted by linear regression. In case of saturation binding experiments with fluorescent labeled ligand 136 in the presence of compound 15, specific binding data were analyzed by a two-parameter equation describing hyperbolic binding (one site-specific binding, GraphPad Prism) to obtain $K_d$ and $B_{\text{max}}$ values. Additionally, specific binding data were normalized to the $B_{\text{max}}$ value, specific binding (%) was plotted against log(concentration of 136) followed by analysis using a four-parameter logistic fit (log(agonist) vs. response, applied constraints: bottom = 0%, top = 100%; GraphPad Prism). Data for the ‘Schild’ plot were obtained from the rightward shift ($\Delta pK_d$) of the saturation isotherm and transformation into log($r-1$) (where $r = 10^{\Delta pK_d}$). Log($r-1$) was plotted against log(concentration of 15) and the data were analyzed by linear regression to obtain the slope and the ‘$pA_2$’ value (intercept with the X axis). Specific binding data from association experiments with 136 were analyzed by a two-parameter equation describing an exponential rise to a maximum (one-phase association, GraphPad Prism) to obtain the observed association rate constant $k_{\text{obs}}$ and the amount of specifically bound 136 at equilibrium ($B_{\text{eq}}$), which was used to calculate specifically bound 136 ($B_t$) in %. Data from dissociation experiments (% specifically bound 136 ($B_t$) plotted over time) were analyzed by a three-parameter equation (one phase decay, GraphPad Prism) to obtain the dissociation rate constant $k_{\text{off}}$. The association rate constant ($k_{\text{on}}$) of 136 was calculated from $k_{\text{obs}}, k_{\text{off}}$ and the concentration of 136 used for the association experiment according to the correlation: $k_{\text{on}} = (k_{\text{obs}}-k_{\text{off}})/[\text{FL}]$. Total binding data from competition binding experiments (determination of the effect of various MR ligands on the equilibrium binding of $[^3H]\text{NMS}$, 135 or 136) were plotted against log(concentration competitor) and analyzed by a four-parameter logistic equation (log(inhibitor) vs. response-variable slope, GraphPad Prism) followed by normalization (100% = ‘top’ of the four-parameter logistic fit, 0% = unspecifically bound fluorescent ligand determined in the presence of 7) and analysis of the normalized data by a four-parameter logistic equation. IC$_{50}$ values were converted to $K_i$ values according to the Cheng-Prusoff equation$^{51}$. Statistical significance was assessed by a one-sample t-test.
4.6. References


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Chapter 5

Summary
5. Summary

In humans, the family of muscarinic acetylcholine receptors (mAChR, MRs) comprises five subtypes (M₁R-M₅R), which are members of the class A GPCR superfamily and mediate the action of the neurotransmitter acetylcholine in the central and peripheral nervous system. For instance, the M₂R, which binds to Gᵢ/o heterotrimeric G-proteins, acts as a presynaptic autoreceptor in the brain and in the periphery. Accordingly, selective M₂R antagonism in the CNS, resulting in enhanced cholinergic transmission, was suggested as an approach to increase cholinergic function in Alzheimer patients. MRs represent important drug targets, however, there is still a need for highly subtype selective MR ligands, as the development of selective agents has been challenging due to the high conservation of the acetylcholine (orthosteric) binding site. Due to the less conserved allosteric binding sites, the dual steric ligand approach, i.e. the design of compounds, which simultaneously address the orthosteric and allosteric binding sites, is considered a promising strategy to develop MR ligands with improved subtype selectivity.

This work was aiming at the synthesis and pharmacological characterization of dibenzodiazepinone-type heterodimeric MR ligands, which were prepared by linking different monomeric MR ligands (agonists, antagonists, orthosteric and allosteric ligands) through various linkers to a pharmacophoric moiety derived from the dibenzodiazepinone DIBA. The synthesis afforded heterodimeric ligands (‘DIBA-xanomeline’, ‘DIBA-TBPB’, ‘DIBA-77-LH-28-1’, ‘DIBA-propantheline’ and ‘DIBA-4-DAMP’) and ‘DIBA-DIBA’-type homodimeric ligands. Equilibrium competition binding studies with [³H]NMS at live CHO cells expressing the respective human MₓR subtype (x = 1-5) revealed a M₂R preference of all dimeric ligands with high M₂R affinities (Kᵢ values: 0.08-5.8 nM). These data demonstrated that the type of the linker (short vs. long, basic vs. non-basic, etc.) and the type of the second pharmacophoric group had only little impact on M₂R binding. As non-DIBA-type monomeric and homodimeric reference compounds exhibited considerably lower M₂R affinities than the synthesized DIBA-derived ligands, the high M₂R affinity of the heterodimeric dibenzodiazepinone-type ligands is most likely conferred by the ‘dibenzodiazepinone’ pharmacophore.

Two tritium-labeled DIBA-derived heterodimeric ligands (‘DIBA-xanomeline’- and DIBA-TBPB’-type) were prepared and characterized by saturation and kinetic binding studies at the hM₂R. Saturation binding experiments showed that these ligands address the orthosteric site of the M₂R. The ‘DIBA-TBPB’-type dimeric radioligand ([³H]115) exhibited a very high M₂R affinity (Kᵢ value 0.13 nM). The investigation of the effect of allosteric MR ligands (gallamine, W84, LY2119620) on the equilibrium binding of [³H]115, and saturation binding studies with [³H]115 in the presence of the allosteric MR ligand W84 (Schild-like analysis) strongly
suggested a competitive mechanism between $[^{3}H]115$ and the investigated allosteric ligand. Consequently, these data revealed that DIBA-derived heterodimeric ligands such as 115 exhibit a dualsteric binding mode at the M$_2$R. Moreover, a series of fluorescently labeled, monomeric DIBA-derived ligands was prepared by conjugation of cyanine dyes or a pyrylum dye via different linkers to the dizenzodiazepinone scaffold. Except for one compound, the fluorescent probes exhibited high M$_2$R affinity ($K_i < 55$ nM). Interestingly, the structure of the fluorophores had less impact on the M$_2$R affinity than the nature of the linker. The fluorescent ligands 135 and 136, bearing red-emitting cyanine dyes attached via linkers with a basic piperazine moiety, exhibited the highest M$_2$R affinity ($K_i$ ca. 1 nM). The M$_2$R preference of the fluorescent ligands was less pronounced compared to the amine-functionalized precursor molecules, i.e. attachment of the lipophilic fluorophores resulted in a decrease in M$_2$R selectivity. Application of 135 and 136 to flow cytometry and high content analysis proved that these new fluorescent probes are suited for such techniques. The fluorescent ligand 136 was identified as a valuable molecular tool for the determination of MR affinities of MR ligands. M$_2$R binding studies with 136 in the presence of allosteric modulators strongly suggested that 136 and structurally related ligands bind simultaneously to both the orthosteric (via the dibenzodiazepinone scaffold) and the ‘common’ allosteric binding site (most likely via the fluorophores) of the M$_2$R, thus exhibiting a dualsteric binding mode, too.

In conclusion, this work afforded new radiolabeled and fluorescently labeled molecular tools for the M$_2$R and suggests dibenzodiazepinone-type MR ligands as an interesting compound class to develop highly selective M$_2$R ligands according to the dualsteric ligand approach.
Chapter 6

Appendix
6. Appendix

6.1. $^1$H-NMR and $^{13}$C-NMR spectra of compounds 41, 46, 53-55, 67, 95-102, 105a, 106-108, 110-120 and 130-136

$^1$H-NMR spectrum (400 MHz, [D$_4$]MeOH) of compound 41.

$^{13}$C-NMR spectrum (100 MHz, [D$_4$]MeOH) of compound 41.
\[\text{\^H-NMR spectrum (300 MHz, CDCl}_3\text{) of compound 46.}\]

\[\text{\^C-NMR spectrum (75 MHz, CDCl}_3\text{) of compound 46.}\]
\(^1\)H-NMR spectrum (300 MHz, CDCl\(_3\)) of compound 53.

\(^{13}\)C-NMR spectrum (75 MHz, CDCl\(_3\)) of compound 53.
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$^1$H-NMR spectrum (300 MHz, CDCl$_3$) of compound 54.

$^{13}$C-NMR spectrum (75 MHz, CDCl$_3$) of compound 54.
\( ^1H \) NMR spectrum (300 MHz, CDCl\(_3\)) of compound 55.

\( ^{13}C \) NMR spectrum (75 MHz, CDCl\(_3\)) of compound 55.
$^1$H-NMR spectrum (600 MHz, [D$_4$]MeOH) of compound 67.

$^{13}$C-NMR spectrum (150 MHz, [D$_4$]MeOH) of compound 67.
1H-NMR spectrum (600 MHz, [D₄]MeOH) of compound 95.

13C-NMR spectrum (150 MHz, [D₄]MeOH) of compound 95.
\(^1\)H-NMR spectrum (600 MHz, [D\(_4\)]MeOH) of compound 96.

\(^{13}\)C-NMR spectrum (150 MHz, [D\(_4\)]MeOH) of compound 96.
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$^1$H-NMR spectrum (600 MHz, [D$_4$]MeOH) of compound 97.

$^{13}$C-NMR spectrum (150 MHz, [D$_4$]MeOH) of compound 97.
\(^1\)H-NMR spectrum (300 MHz, [D\textsubscript{4}]MeOH) of compound \textit{98}.

\(^{13}\)C-NMR spectrum (75 MHz, [D\textsubscript{4}]MeOH) of compound \textit{98}.
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$^1$H-NMR spectrum (300 MHz, [D$_4$]MeOH) of compound 99.

$^{13}$C-NMR spectrum (75 MHz, [D$_4$]MeOH) of compound 99.
$^1$H-NMR spectrum (300 MHz, [D$_4$]MeOH) of compound 100.

$^{13}$C-NMR spectrum (75 MHz, [D$_4$]MeOH) of compound 100.
$^1$H-NMR spectrum (300 MHz, [D$_4$]MeOH) of compound 101.

$^{13}$C-NMR spectrum (75 MHz, [D$_4$]MeOH) of compound 101.
\(^1\)H-NMR spectrum (300 MHz, [D₄]MeOH) of compound 102.

\(^{13}\)C-NMR spectrum (75 MHz, [D₄]MeOH) of compound 102.
$^1$H-NMR spectrum (600 MHz, [D$_4$]MeOH) of compound 105a.

$^{13}$C-NMR spectrum (75 MHz, [D$_4$]MeOH) of compound 105a.
$^1$H-NMR spectrum (600 MHz, [D$_4$]MeOH) of compound 106.

$^{13}$C-NMR spectrum (150 MHz, [D$_4$]MeOH) of compound 106.
$^{1}H$-NMR spectrum (600 MHz, [D$_4$]MeOH) of compound 107.

$^{13}C$-NMR spectrum (150 MHz, [D$_4$]MeOH) of compound 107.
\[ ^1H\text{-NMR spectrum (600 MHz, [D\textsubscript{4}]MeOH)} \text{ of compound 108.} \]

\[ ^{13}C\text{-NMR spectrum (150 MHz, [D\textsubscript{4}]MeOH)} \text{ of compound 108.} \]
$^1$H-NMR spectrum (600 MHz, [D₄]MeOH) of compound 110.

$^{13}$C-NMR spectrum (150 MHz, [D₄]MeOH) of compound 110.
$^1$H-NMR spectrum (600 MHz, [D$_4$]MeOH) of compound 111.

$^{13}$C-NMR spectrum (150 MHz, [D$_4$]MeOH) of compound 111.
\(^1\)H-NMR spectrum (600 MHz, [D\textsubscript{4}]MeOH) of compound 112.

\(^{13}\)C-NMR spectrum (150 MHz, [D\textsubscript{4}]MeOH) of compound 112.
$^1$H-NMR spectrum (600 MHz, [D$_4$]MeOH) of compound 113.

$^{13}$C-NMR spectrum (150 MHz, [D$_4$]MeOH) of compound 113.
$^1$H-NMR spectrum (600 MHz, [D$_4$]MeOH) of compound 114.

$^{13}$C-NMR spectrum (150 MHz, [D$_4$]MeOH) of compound 114.
$^1$H-NMR spectrum (600 MHz, [D$_6$]DMSO) of compound 115.

$^{13}$C-NMR spectrum (150 MHz, [D$_6$]DMSO) of compound 115.
$^{1}H$-NMR spectrum (600 MHz, $[D_4]$MeOH) of compound 116.

$^{13}C$-NMR spectrum (150 MHz, $[D_4]$MeOH) of compound 116.
$^1$H-NMR spectrum (600 MHz, [D$_4$]MeOH) of compound 117.

$^{13}$C-NMR spectrum (150 MHz, [D$_4$]MeOH) of compound 117.
\(^1\)H-NMR spectrum (600 MHz, [D\(_4\)]MeOH) of compound 118.

\(^{13}\)C-NMR spectrum (150 MHz, [D\(_4\)]MeOH) of compound 118.
\textbf{Chapter 6}

1\textsuperscript{H}-NMR spectrum (600 MHz, \textit{[D}_4\textit{]}\textit{MeOH}) of compound 119.

1\textsuperscript{3}C-NMR spectrum (150 MHz, \textit{[D}_4\textit{]}\textit{MeOH}) of compound 119.
$^1$H-NMR spectrum (600 MHz, [D$_4$]MeOH) of compound 120.

$^{13}$C-NMR spectrum (150 MHz, [D$_4$]MeOH) of compound 120.
\(^1\)H-NMR spectrum (600 MHz, MeOH-d<sub>4</sub>) of compound 130.

\(^1\)H-NMR spectrum (600 MHz, MeOH-d<sub>4</sub>) of compound 131.
$^1$H-NMR spectrum (600 MHz, MeOH-$d_4$) of compound 132.

$^1$H-NMR spectrum (600 MHz, MeOH-$d_4$) of compound 133.
1H-NMR spectrum (600 MHz, MeOH-d4) of compound 134.

1H-NMR spectrum (600 MHz, MeOH-d4) of compound 135.


RP-HPLC analysis (purity control) of 41 and 46.
RP-HPLC analysis (purity control) of 53 and 54.

RP-HPLC analysis (purity control) of 55 and 67.

RP-HPLC analysis (purity control) of 95 and 96.

RP-HPLC analysis (purity control) of 97 and 98.
RP-HPLC analysis (purity control) of 99 and 100.

RP-HPLC analysis (purity control) of 101 and 102.

RP-HPLC analysis (purity control) of 105a and 106.

RP-HPLC analysis (purity control) of 107 and 108.
RP-HPLC analysis (purity control) of 110 and 111.

RP-HPLC analysis (purity control) of 112 and 113.

RP-HPLC analysis (purity control) of 114 and 115.

RP-HPLC analysis (purity control) of 116 and 117.
Chapter 6

RP-HPLC analysis (purity control) of 118 and 119.

RP-HPLC analysis (purity control) of 120 and 130.

RP-HPLC analysis (purity control) of 131 and 132.
RP-HPLC analysis (purity control) of 133 and 134.

![Graph](image)

RP-HPLC analysis (purity control) of 135 and 136.

6.3. Abbreviations

α intrinsic activity or selectivity factor  
A agonist  
abs absolute  
AC adenyl cyclase  
aq aqueous  
atm atmosphere  
Boc tert-butoxycarbonyl  
Boc₂O di-tert-butyl dicarbonate  
Bq Becquerel  
Bₘₐₓ the maximal specific binding of a ligand  
BRET bioluminescence resonance energy transfer  
brs broad singlet  
BSA bovine serum albumin  
[Ca²⁺]ᵢ intracellular calcium ion concentration  
calcd. calculated  
cAMP cyclic 3', 5'-adenosine monophosphate  
CH₂Cl₂ dichloromethane  
CHCl₃ chloroform  
CH₃CN acetonitrile  
CHO-cells Chinese hamster ovary cells  
Cⁱ curie  
CNS central nervous system  
COSY correlated spectroscopy  
cpm counts per minute  
d day(s) or doublet  
DAG diacylglycerol  
δ chemical shift  
DCC N,N'-dicyclohexylcarbodiimide  
DCM dichloromethane  
dd doublet of doublets  
DIPEA diisopropylethylamine  
DMAP 4-dimethylaminopyridine  
DMF dimethylformamide  
DMSO dimethylsulfoxide
DMSO-d₆  per-deuterated dimethylsulfoxide

EC₅₀  agonist concentration which induces 50 % of the maximum response

EDC  N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride

eq  equivalents

EtOAc  ethylacetate

Et₂O  diethylether

EtOH  ethanol

FACS  fluorescence activated cell sorter

FCS  fetal bovine serum

Fl-1, Fl-2, Fl-3, Fl-4  fluorescence channels (Flow cytometer)

FRET  fluorescence resonance energy transfer

G  G-Protein

GDP  guanosine diphosphate

GTP  guanosine triphosphate

GPCR  G-protein coupled receptor

h  hour(s) or human

HCl  hydrochloric acid

HMBC  heteronuclear multiple bond correlation

HSQC  heteronuclear single quantum correlation

HOBT  1-Hydroxybenzotriazole hydrate

HPLC  high-performance liquid chromatography

HRMS  high resolution mass spectrometry

Hz  hertz

IC₅₀  radioligand binding assay: ligand concentration inhibiting the binding of a radioligand by 50 %

IP₃  inositol-1,4,5-trisphosphate

IR  infrared

J  coupling constant

k  capacity factor

K₆  dissociation constant (functional assay)

KBr  potassium bromide

K₂CO₃  potassium carbonate

K₆  dissociation constant (saturation binding)

KH₂SO₄  potassium bisulfate

K₆  dissociation constant (competition binding)

kₐ  observed rate constant

kₐ  dissociation rate constant

kₐ  association rate constant

L  liter

LiAlH₄  Lithiumaluminiumhydrid

L15  Leibovitz medium without phenol red

m  multiplet

M  molar (mol/L)

mAU  milli absorbance units

MeCN  acetonitrile

MeOH  methanol

MeOH-d₄  per-deuterated methanol

MeI  methyl iodide

mol  mole (s)

min  minute(s)

μ  micro
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>mp</td>
<td>melting point</td>
</tr>
<tr>
<td>MR</td>
<td>Muscarinic receptor</td>
</tr>
<tr>
<td>Mx,R</td>
<td>Muscarinic Mx receptor (x = 1, 2, 3, 4, 5)</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>n</td>
<td>nano or amount of substance</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>sodium bicarbonate</td>
</tr>
<tr>
<td>NaI</td>
<td>sodium iodide</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>sodium sulfate</td>
</tr>
<tr>
<td>NEt₃</td>
<td>triethylamine</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>OBD</td>
<td>orthosteric binding domain</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>petroleum ether</td>
</tr>
<tr>
<td>pEC₅₀</td>
<td>negative decadic logarithm of the molar concentration of the agonist causing 50% of the maximal response</td>
</tr>
<tr>
<td>Ph</td>
<td>phenyl</td>
</tr>
<tr>
<td>Ph₃P</td>
<td>triphenylphosphine</td>
</tr>
<tr>
<td>PIP₂</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLCβ</td>
<td>phospholipase Cβ</td>
</tr>
<tr>
<td>pKᵃ</td>
<td>negative decadic logarithm of the dissociation constant (functional assay)</td>
</tr>
<tr>
<td>pKi</td>
<td>negative decadic logarithm of the dissociation constant (competition binding assay)</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>Py</td>
<td>pyridyl or pyrylium</td>
</tr>
<tr>
<td>q</td>
<td>quartet</td>
</tr>
<tr>
<td>ref</td>
<td>reference</td>
</tr>
<tr>
<td>Rₙ</td>
<td>retardation factor</td>
</tr>
<tr>
<td>RGS</td>
<td>regulator of G-protein signaling</td>
</tr>
<tr>
<td>RP</td>
<td>reversed phase</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>rt</td>
<td>room temperature</td>
</tr>
<tr>
<td>s</td>
<td>singulet</td>
</tr>
<tr>
<td>sat.</td>
<td>saturated</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>t</td>
<td>triplet</td>
</tr>
<tr>
<td>t₀</td>
<td>dead time</td>
</tr>
<tr>
<td>TBDPS</td>
<td>tert-butyldiphenysily</td>
</tr>
<tr>
<td>TBTU</td>
<td>2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>TMS</td>
<td>trimethylsilyl</td>
</tr>
<tr>
<td>tᵣ</td>
<td>retention time</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolett</td>
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</table>