Dimeric ligands for GPCRs involved in human reproduction: synthesis and biological evaluation

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Cover: Topograph obtained by atomic-force microscopy showing the regular rearrangement of rhodopsin in the native disc membrane. Picture adapted from Fotiadis, D.; Liang, Y.; Filipek, S.; Saperstein, D. A.; Engel, A.; Palczewski, K. *Nature* **2003**, *421*, 127-128.

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List of Abbreviations

5-HT_2R	serotonin receptor type 2
[α]	specific rotation [(deg·mL)/(g·dm)]
Å	angstrom(s)
Abu	aminobutyric acid
AC	adenylyl cyclase
Ac	acetyl
ADP	adenosine diphosphate
AIBN	2, 2'-azobis(isobutyronitrile)
Ahx	aminohexanoic acid
Anhyd	anhydrous
Ar	aryl
Aq	aqueous
ATP	adenosine triphosphate
Azp	4-azidoproline
B_2R	bradykinin receptor type 2
β_2AR	β2-Adrenergic receptor
Bn	benzyl
Boc	*tert*-Butyloxycarbonyl
BOP	benzotriazol-1-yloxytri (dimethylamino)phosphonium hexafluorophosphate
br	broad (*NMR*)
BRET	bioluminescence resonance energy transfer
calcd	calculated
cAMP	cyclic adenosine mono-phosphate
CCR	chemokine receptors
CD	circular dichroism
$CDCl_3$	deuterated chloroform
CDI	carbonyldiimidazole
cDNA	complementary deoxyribonucleic acid
CHO cells	Chinese Hamster Ovary cells
cm^{-1}	wavenumber(s)
conc	concentration
CRE	cAMP response element
CV	column volume
δ	chemical shift (*NMR*)
d	doublet (*NMR*)
d	day(s)
DCE	dichloroethane
DCM	dichloromethane
dd	doublet of doublets (*NMR*)
ddd	double doublet of doublets (*NMR*)
deg	degrees
DiPEA	diisopropylethylamine
DHQ	dihydroquinoline
DMAP	4-(*N*,*N*-dimethylamino)-pyridine
DMEM	dulbecco's modified Eagle's medium
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxide
EC	effective concentration
Eq	molar equivalents
ER	endoplasmatic reticulum
ESI	electronspray ionization
Et	ethyl
EtOAc	ethyl acetate
(E)YFP	(enhanced) yellow fluorescence protein
Fmoc	9-Fluorenylmethoxycarbonyl
FRET	fluorescence resonance energy transfer
FSH(R)	follicle stimulating hormone (receptor)
$GABA_B$	γ-aminobutyric acid type B
GAP	GTPase accelerating proteins
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GnRH(R)	gonadotropin-releasing hormone (receptor)
GPCR	G-protein coupled receptor
GpHR	glycoprotein hormone receptor
GRK	G-protein receptor kinases
GTP	guanosine triphospate

h	hour
HA	hemagglutinin
hCG	human chorionic gonadotropin
HCTU	2-(6-chloro-1H-benzo triazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
Hyp	hydroxyproline
IC	inhibitory concentration
i	iso
IP_3	inositol 1,4,5-triphosphate
IR	infra red
J	coupling constant
LC-MS	liquid chromatography – mass spectrometry
LH(R)	luteinizing hormone (receptor)
LHA	luteinizing hormone receptor agonist
LMW	low molecular weight
m	multiplet (*NMR*)
M	molar (moles per liter)
Me	methyl
MeO	methoxy
MeOH	methanol
MHz	megahertz
min	minute(s)
MS	mass spectrometry
M_2R	muscarinic receptor type 2
m/z	mass-to-charge ratio
NBS	*N*-bromosuccinimide
n.a.	not active
n.d.	not determined
NMM	*N*-methylmorpholine
NMP	*N*-methylpyrrolidone
NMR	nuclear magnetic resonance
NOE(SY)	nuclear Overhauser effect (spectroscopy)
nu	nucleophile
obsd	observed
on	overnight
PBS	phosphate-buffered saline
PE	petroleum ether
PEG	polyethylene glycol
Pip	4-pipecolic acid
PKA	protein kinase A
PLC	phospholipase C
ppm	parts per million (*NMR*)
Pro	proline
q	quartet *(NMR*)
quant	quantitative
R_f	retention factor (*TLC*)
Rluc	*renilla* luciferase protein
RP	reversed phase
rt	room temperature
s	singulet (*NMR*)
SAR	structure activity relationships
sat	saturated
SPPS	solid phase peptide synthesis
t	tertiary
t	triplet (*NMR*)
tert	tertiary
θ	molar ellipticity ($deg \cdot cm^2 \cdot dmol^{-1}$)
THF	tetrahydrofuran
THQ	tetrahydroquinoline
TIS	triisopropylsilane
TLC	thin layer chromatography
TFA	trifluoroacetic acid
TM	transmembrane
t_R	retention time (in chromatography)
Trz	triazole
TSH(R)	thyroid stimulating hormone (receptor)
v/v	volume per unit volume
VFTM	venus flytrap module
V_2R	vasopressin receptors type 2

Chapter 1

General introduction

G-protein coupled receptors

The superfamily of G-protein coupled receptors (GPCRs) comprises structurally conserved membrane proteins that are characterized by a common seven-helical transmembrane (7TM) motif.[1] The receptor helices are interconnected by a set of alternating intracellular- and extracellular loops. GPCRs exert their primary role, signal transduction across the cellular membrane, under the influence of distinct types of (endogenous) ligands, including photons, ions, heterocyclic and peptidic molecules and proteins.[2] GPCRs play a crucial role in many biological processes and the availability of small molecule GPCR agonists and antagonists explains why GPCRs are among the most investigated target classes to date.[3]

GPCRs are intracellularly bound to a G-protein as depicted in Figure 1.[4] The hetero-trimeric G-protein composes of an α, β, and γ domain and adopts an inactive state when the Gα subunit is bound to guanine diphosphate (GDP, hence the name G-protein). Activation of the G-protein occurs by binding of an extracellular ligand to the receptor.[5] The GPCR then undergoes a conformational change after which the Gα subunit generates a high-affinity binding site for

Box 1: General terms used to describe drug action

Agonist: A ligand that binds to a receptor inducing a conformational change resulting in a biological response.
Partial agonist: A ligand that binds to the receptor but only has a partial effect on the receptor compared to the full agonist.
Inverse agonist: A ligand that reduces the constitutive activity of a receptor.
Antagonist: A ligand that does not induce a signal when bound to the receptor alone but reduces agonist-mediated response. The effect of the antagonist can be overcome by increasing the concentration of agonist.
Insurmountable antagonist: An antagonist of which the effect can not be overcome by increasing the concentration of agonist. Often insurmountable antagonists have extreme slow dissociation rates compared to competitive antagonists.
Allosteric modulator: A ligand that increases or decreases the action of an (primary or orthosteric) agonist or antagonist by binding to a distinct (allosteric) site on the receptor.
Affinity: The property of a ligand to bind to a receptor.
Potency: A measure of the concentration of a drug at which it is effective.
Efficacy: The property that allows ligands, once bound, to produce a response.

guanosine triphosphate (GTP) and exchanges GDP for GTP. The receptor thereby serves as a guanine nucleotide exchange factor (GEF) for G-proteins. The activated GTP-bound Gα-subunit readily dissociates from the receptor as well as from the Gβ,γ subunits after which the separate subunits associate with other intracellular effectors such as adenylyl cyclase (AC), phospholipase C or calcium ion channels.[4] The autocatalytic properties of the Gα subunit hydrolyze GTP into GDP (accelerated by GTPase accelerating proteins or GAPs) and the inactive GDP-bound Gα re-associates with the Gβ,γ subunits and the GPCR to undergo another cycle of activation. In the absence of ligand, GPCRs also equilibrate between a number of active and inactive conformers.[6] This natural equilibration leads to activation of the G-proteins and therefore most GPCRs have some intrinsic activity in the absence of ligand.

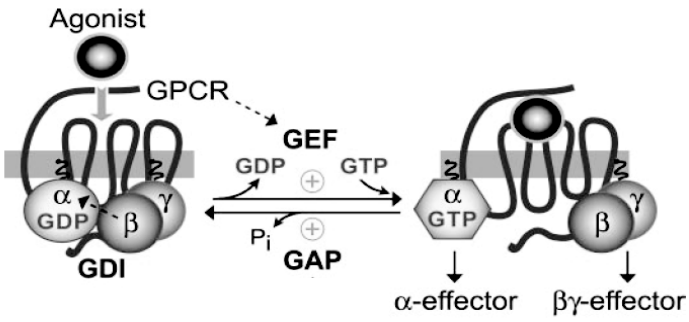


Figure 1. General overview of the intracellular mechanism of action of G-protein signaling.

In order to obtain receptor signaling specificity, four types of G-proteins are distinguished (further detailed in Box 2).[7] The G-proteins are classified, based on their properties to activate or inhibit specific intracellular effector types. The hypothesis that a GPCR activates only one specific G-protein was long acknowledged. However, several recent literature reports evidenced that a single GPCR may be associated with multiple G-protein signaling pathways.[8] For example, mutagenesis studies on the β-adrenergic receptor revealed that both G_s and $G_{i/o}$ proteins are involved in receptor activation[9] and for some GPCRs it was found that all four types of G-proteins may be involved.[10]

Box 2: Types of G-proteins and signaling[7]

The specificity of GPCR mediated signaling is dependent on the nature of the G-protein. Currently, four families of Gα-proteins have been classified, namely the G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$. The proteins differ primarily in effector recognition but share a similar mechanism of activation. Receptors that are coupled to stimulatory **G_s proteins** stimulate a membrane-associated protein called adenylate cyclase (AC) that in turn stimulates the production of cAMP from ATP. cAMP activates protein kinase A (PKA) that phosphorylates other downstream (and upstream) targets. The **$G_{i/o}$ proteins** in contrary inhibit the formation cAMP by AC. The **G_q proteins** activate phospholipase C (PLC). This enzyme cleaves phosphoinositol PIP_2 into two second messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3 in turn triggers the release of calcium from intracellular compartments. The **$G_{12/13}$ proteins** are involved in the regulatory role of Rho guanine exchange factors that in turn activate Rho small GTPases. In some regulatory mechanisms the Gβ,γ complex also activates downstream proteins in the cell.

The activity of GPCRs is intracellularly controlled by a set of complex, yet not fully understood regulatory mechanisms. One of the best-studied mechanisms is a phenomenon called desensitization, that is, the reduction of a signal even in the continuing presence of ligand.[11,12] Desensitization often occurs after the phosphorylation of the receptor by kinases that are activated by the receptor itself (for example PKA via the G_s pathway, box 3) or by a specific class of G-protein receptor kinases (GRKs) that solely phosphorylate activated GPCRs. In some cases, intracellular proteins called β-arrestins bind the phosphorylated GPCRs and thereby sterically hinder G-proteins to couple to their receptor.[13,14] Some GPCRs are dephosphorylated by phosphatases that are located in intracellular compartments and for this to happen these GPCRs must find their way into these intracellular compartments via internalization. GPCR-mediated signaling is also controlled by GPCR degradation, after internalization, in endosomal compartments.

GPCR dimerization

GPCRs were initially considered as monomeric entities that could bind to one individual G-protein and one ligand molecule. This classical view was heavily challenged during the years by the emerging body of evidence supporting the idea that GPCRs can exist and may also function in dimeric or oligomeric assemblies.[15-19]

Already more than two decades ago, several studies raised the hypothesis that GPCR dimerization may play a role in receptor signaling. For the β-adrenergic receptor it was observed that binding of one ligand diminishes the binding of a second ligand to a receptor (negative cooperativity).[20] Alternatively, it was shown for the gonadotropin-releasing hormone receptor (GnRHR) that an agonistic response was induced when two peptide antagonists were interconnected by a monoclonal antibody. From these results it was suggested that, at least for the GnRHR, an antagonist becomes an agonist when it is capable of bridging two receptors with a distance between 15 and 150 Å.[21]

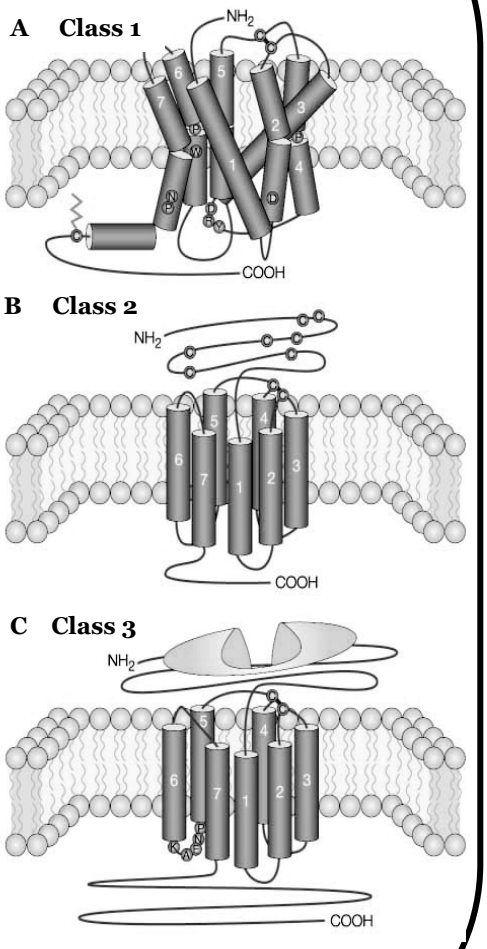
Box 3: Classification of GPCRs[2,3]

The G protein-coupled receptors are divided in three main subclasses, based on their primary sequence.

Class 1 (or class A) is the largest (90%) and most well-studied. It includes the rhodopsin and adrenergic receptors and is characterized by several conserved amino acids in the transmembrane domain (dots, Figure a). Two cysteine residues in extracellular loops 1 and 2 usually form a disulfide bridge, connecting the two loops. Besides, a palmitoylated cysteine resides in the carboxy-terminal tail. Most class 1 receptors have a short extracellular N-terminus. Their ligands, low molecular weight compounds, bind inside the 7TM domain. However, some class 1 receptors bind larger molecules, such as glycoproteins, to the N-terminus and the extracellular loops.

The 53 currently known members of the **class 2** (or class B) GPCRs have a large N-terminal domain, containing many cysteine amino acids that can form disulfide bonds. The receptors bear little resemblance to the class 1 receptors. Their ligands, hormones and peptides, bind to the N-terminus of these receptors.

Only 19 **class 3** (or class C) GPCRs have been identified so far in humans. Among these are the receptors for metabotropic glutamate, γ-aminobutyric acid (GABA) and calcium. These receptors are characterized by long N- and C-terminal domains. The N-terminus contains a so-called venus flytrap module (VFTM) that is involved in ligand binding. Figure adapted from reference 16.

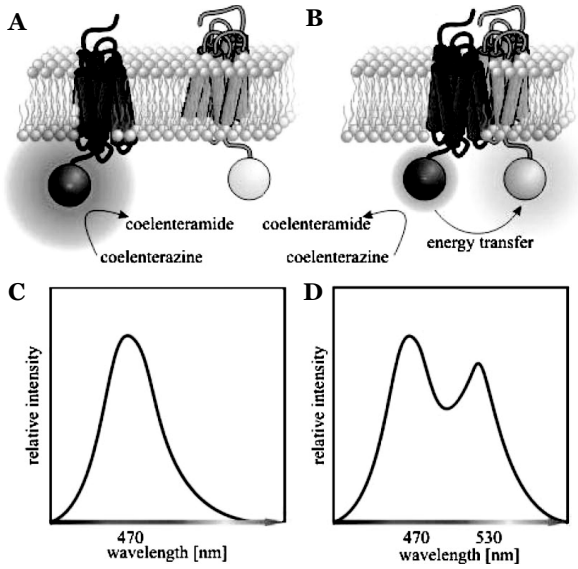


In the following years, more support for receptor dimerization was provided by biochemical techniques such as co-immunoprecipitation and fluorescence energy transfer experiments. The first evidence of GPCR dimerization by co-immunoprecipitation was demonstrated for the β_2-adrenergic receptor (β_2AR).[22] This receptor was equipped with either an influenza hemagglutinin (HA)- or a myc-epitope and coexpressed. After immunoprecipitation with an anti-myc antibody and Western blotting analysis, the HA-β_2AR fusion protein could be detected with an anti-HA-antibody. This observation evidenced that β_2AR may exist as homodimers in the cell membrane. To further confirm the results and as a control, a HA-tag was also incorporated in a M_2-muscarinic receptor (HA-M_2R) that was then coexpressed with myc-β_2AR. For this combination, receptor dimerization was not expected and after co-immunoprecipitation with and anti-myc antibody, no HA-M_2R could be detected with an anti-HA-antibody. In the following years many receptor dimers have been identified by the use of this technique ranging from homodimers (for example, dopamine and δ-opioid receptors), heterodimers of two closely related GPCRs ($GABA_{B1}$/$GABA_{B2}$ receptors and δ-/μ-opioid receptors) and more distantly related heterodimers (δ-opioid and β_2-adrenergic receptors).[18,19]

With the developments based on resonance energy transfer (RET) techniques, additional evidence for receptor dimerization arose (see Box 4 for further details, reviewed in reference 23). RET involves the expression of GPCRs equipped with fluorescence labels that differ in resonance properties on the intracellular C-termini of the receptor. When the receptors are coexpressed and in close proximity (<100 Å) the energy of one of the labels is transferred to the other causing a change in the fluorescence properties of the labels. The RET techniques have the benefit that they can be used in living cells so that GPCR dynamics may be studied in more detail. For example, β_2-adrenergic receptors (β_2AR) in which one is conjugated to a *renilla* luciferase protein (Rluc) and one to a green fluorescent protein (GFP), showed formation of homodimers in the absence of ligand (constitutive dimers).[24] In addition, when a selective β_2AR agonist was added to the cells, an increase in RET signal was observed, indicating that additional receptor dimers were formed.

Probably the most direct evidence for GPCR dimerization came from studies concerning the class C metabotropic $GABA_{B1}$ and $GABA_{B2}$ receptors.[25-27] These receptors proved not functional when expressed independently, but became active when expressed simultaneously. Further studies revealed that the $GABA_{B1}$ receptor, when expressed alone, retained in the endoplasmatic reticulum (ER) because of the ER-retention motif present in its carboxy terminus. $GABA_{B2}$ did not retain in the ER but proved not functional when expressed on the cell surface alone. When both receptors were coexpressed, the $GABA_{B2}$ shielded the retention signal of the $GABA_{B1}$ receptor thereby allowing the complex to reach the cell membrane. The receptor heterodimer also proved functional and it was shown that the $GABA_{B2}$ receptor binds the endogenous ligand while $GABA_{B1}$ binds the G-protein.[28] The phenomenon of such receptor 'cross-talk' was further demonstrated in another study involving the somatostatin receptor.[29] Here, one receptor mutant was developed as such it could not bind to the endogenous ligand while the other mutant could not couple to the G-protein. The mutant receptors proved not functional when expressed alone but signal transduction was restored when both receptors were coexpressed.

Box 4: BRET and FRET



Schematic representation of the application of bioluminescence resonance energy transfer (BRET) to detect GPCR dimerization. The protein *Renilla* luciferace (Rluc) is fused to a GPCR of interest. Rluc degrades the substrate coelenterazine to coelenteramide thereby emitting light with a specific wavelength of 470 nm (Figure A and C). The Rluc fused receptor is coexpressed with a receptor that is linked to an energy acceptor (such as enhanced yellow fluorescence protein, EYFP). When the two receptors are in close proximity (that is <100 Å, Figure B), the energy that results from the degradation of coelenterazine by Rluc is absorbed by EYFP resulting in an additional emission of light of 530 nm that is characteristic for EYFP (D). Fluorescence RET (FRET) is similar to BRET but here the energy donor is a fluorescence protein (such a cyan fluorescence protein) instead of a bioluminescence protein. Figure adapted from reference 56

The first structural evidence that GPCRs may exist in dimers was obtained from the crystallized extracellular ligand-binding domain of the class C metabotropic glutamate receptor.[30] Here, the crystals showed disulphide linked homodimers when co-crystallized with glutamate but also in unliganded form. In 2003, an atomic force microscopy study of the rod outer segments showed that the GPCR rhodopsin was organized in a dimeric and oligomeric fashion.[31,32] Together with the reported crystal structures of rhodopsin[33] it was found that this GPCR exists in a dimeric or even oligomeric fashion (Figure 2). Very recently, the crystal structures of β_1AR[34] and β_2AR[35] became available. Both structures proved to be monomeric when co-crystallized with an antagonist (for β_1AR) or an inverse-agonist (for β_2AR) when the receptor was in complex with a monoclonal antibody to facilitate crystallization. In another study, where the third intracellular loop of β_2AR was replaced by a T_4 lysozyme entity, a multilayered arrangement of the receptors was observed.[36] Still, no crystal structure has become available for a GPCR that is co-crystallized with a diffusible agonist (and thus present in the active state) that therefore impedes a conclusive picture on the exact organization of GPCRs by this technique.

Figure 3 shows schematically the potential roles of receptor dimerization during a GPCR life cycle.[37] Receptor dimerization may occur upon maturation of the GPCRs in the endoplasmatic reticulum (ER).[38] This was clearly shown for the $GABA_{B1}$ and $GABA_{B2}$ receptors that need to form

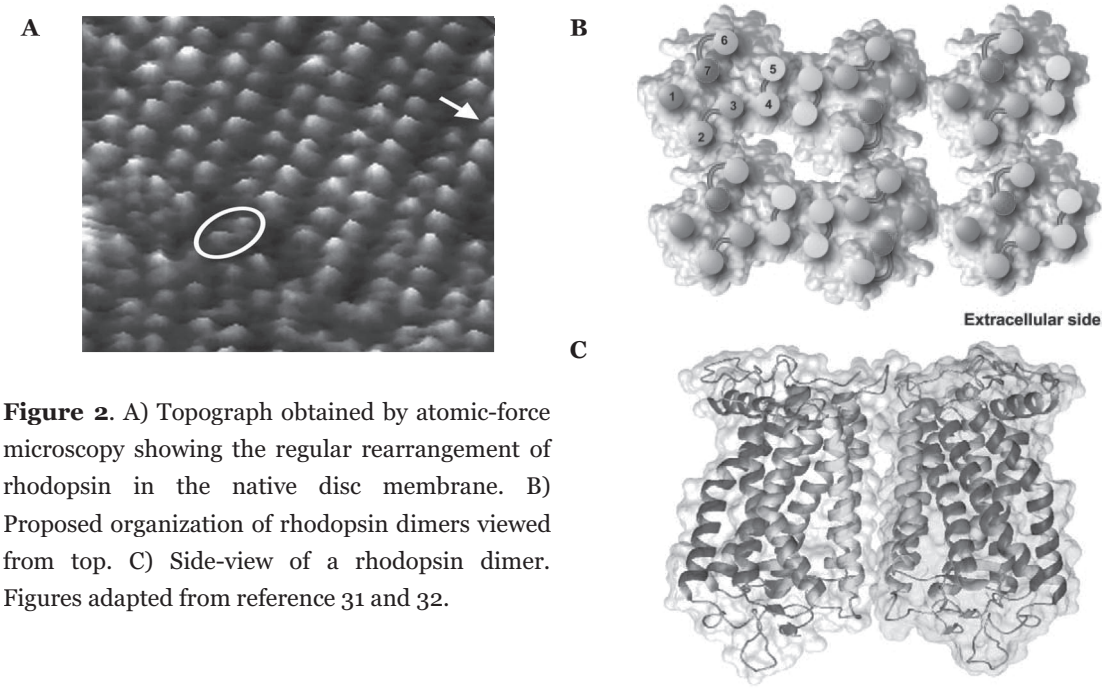


Figure 2. A) Topograph obtained by atomic-force microscopy showing the regular rearrangement of rhodopsin in the native disc membrane. B) Proposed organization of rhodopsin dimers viewed from top. C) Side-view of a rhodopsin dimer. Figures adapted from reference 31 and 32.

heterodimers in order to reach the cell-surface (as described above).[25-27] Fluorescence resonance energy transfer (FRET) experiments further demonstrated that various other GPCRs exist in a dimeric form in the ER as a continuous RET was observed for receptors that were equipped with fluorescent proteins. It was suggested that receptor dimerization may be a prerequisite to pass quality-control checkpoints by chaperone proteins during the biosynthesis.[38]

When one assumes that GPCRs dimerize during biosynthesis and subsequent folding processes, it is also plausible to assume that the receptors are transported to the cell-membrane in a dimeric fashion. Indeed, FRET experiments confirm a constitutive FRET activity for certain receptors at the cell-membrane (Box 4).[18,19] In addition, for some GPCRs, an increase in FRET signal was observed when an agonist was added to the receptor. This indicates that agonists may induce GPCR dimerization. On the contrary, it has also been reported that upon addition of an agonist a decrease in FRET was observed, which indicates agonist-induced dissociation of the GPCR dimer (reviewed in reference 37). Disregarding the fact that agonist-induced dimerization may take place for some receptors different pharmacologic and activity profiles have been shown for certain dimeric receptors when compared to the monomers. For example, the δ- and κ-opioid receptor heterodimers play a role in pharmacological diversity.[39] When separate endogenous ligands were added to heterodimeric δ/κ-opioid receptors, a low binding affinity for the ligands was observed. When the ligands were added simultaneously, the high binding affinity was restored. This indicates a positive cooperativity of the receptors for ligand binding.

Regarding intracellular signaling, several studies have shown that heterodimeric receptors that are coexpressed exhibit different G-protein activation mechanisms compared to GPCRs that are individually expressed. For example, coexpressed CCR2 and CCR5 chemokine receptors appear to induce $G_{q/11}$ signaling which was not observed when CCR5 or CCR2 were homogeneously expressed.[40] Similar results were also observed for the μ- and δ-opioid receptors.[41,42]

The complexity of GPCR mediated signaling and receptor dimerization was further emphasized by chemical cross-linking of the leukotriene B_4 receptor. It was found that two receptor proteins were linked to one G-protein (trimer).[43] This 2:1 receptor-G-protein stoichiometry was also found for the 5-HT_{2c} receptor. Here, receptor mutation studies revealed that a receptor dimer binds two ligands and one G-protein.[44] In contrast, several other groups validated the 1:1 stoichiometry of receptor and G-protein that argued the concept of two receptors and one G-protein.[45] For example, it was shown that one rhodopsin receptor was sufficient to fully activate the G-protein.[46] However, when it is true that two receptors are involved in binding to one G-protein, this may explain why different G-proteins and thus signaling profiles are involved in receptor (hetero)dimers compared to receptor monomers.

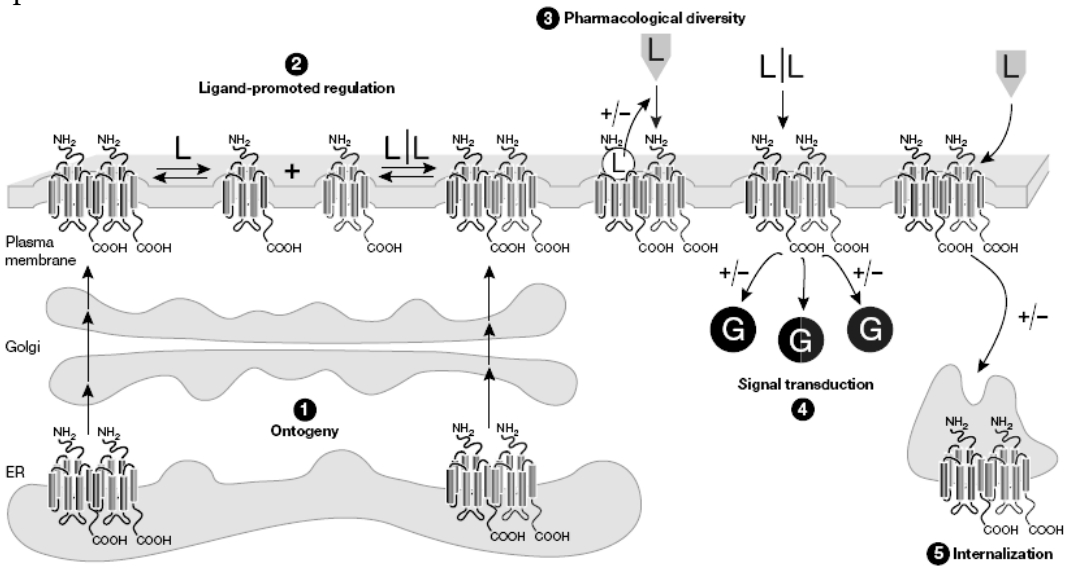
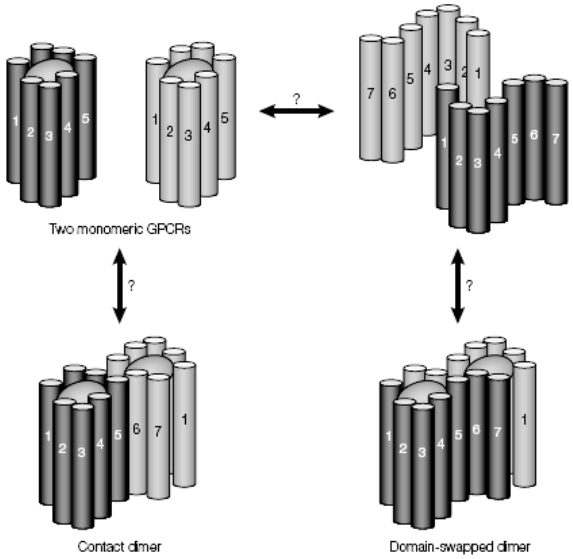


Figure 3. Potential roles of G-protein-coupled receptor (GPCR) dimerization during the GPCR life cycle. (1) In some cases, dimerization has been shown to have a primary role in receptor maturation and allows the correct transport of GPCRs from the endoplasmic reticulum (ER) to the cell surface. (2) Once at the cell membrane, dimers might become the target for dynamic regulation by ligand binding. (3) It has been proposed that GPCR heterodimerization leads to both positive (+) and negative (−) ligand binding cooperativity, as well as (4) potentiating (+)/attenuating (−) signaling or changing G-protein selectivity. (5) Heterodimerization can promote the co-internalization of two receptors after the stimulation of only one of the receptors. Alternatively, the presence of a GPCR that is resistant to agonist-induced endocytosis, within a heterodimer, can inhibit the internalization of the complex. G = G protein; L = ligand. Figure adapted from reference 37.

A final process in which GPCR dimerization may play a role is receptor desensitization and internalization. It was recently demonstrated that the V_{1a} and the V_2 vasopressin receptors are internalized as a stable heterodimer by a β-arrestin mediated process.[47] Both V_{1a} and V_2 have different β-arrestin mediated pathways. While agonist-induced V1a internalization is rapidly followed by dissociation from β-arrestin and retransportation to the cell membrane, V_2 does not dissociate from β-arrestin and accumulates in the endosomes. It appeared that promotion of the V_{1a}/V_2 heterodimer with a V_2 agonist leads to the V_2 internalization pathway, while promotion with a V_{1a} agonist leads to the V_{1a} internalization pathway. This clearly demonstrates that receptor dimerization as well as the nature of the agonist determines the fate of the internalized V_{1a}/V_2 receptors.

Box 5: Receptor dimer formation

In recent years it has been a considerable matter of debate in what fashion GPCRs may form dimers. Mutation and computational studies support the model of domain-swapped dimers, in which helices 6 and 7 are exchanged. It has been proposed that domain-swapping may occur to rescue the activity of deficient receptors. This model is in contrast with rhodopsin oligomers that constitute as hydrophobic bundles that are unlikely to undergo rearrangements. In addition, cross-linking studies in which a peptide was covalently bound to transmembrane helices 1 and 7 showed that these were from the same receptor, thus making domain swapping highly unlikely. Figure adapted from reference 16.



Gonadotropin-Releasing Hormone Receptor

The gonadotropin-releasing hormone receptor (GnRHR) is a well-studied GPCR, with the decapeptidic gonadotropin-releasing hormone (GnRH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) acting as its endogenous ligand.[48,49] GnRH is secreted by the hypothalamus, a process which, in turn, is regulated by the GPR54 receptor.[50] GnRH operates as a key regulator in mammalian sexual maturation and reproductive functions. After binding of GnRH to the GnRHR, the release of gonadotropins (LH and FSH) in anterior pituitary gonadotropes is stimulated (further detailed in Box 6). The major signal transduction route for the GnRHR is via the G_q protein which induces release of intracellular calcium. However, recent reports also suggest the involvement of G_s and G_i in certain cell-lines of which stimulation is agonist-dependent.[48] It is thought that this switch is important in regulating the pulsatile secretion of GnRH.[51] GnRH and its peptide analogues are currently used in modulating gonadotropin and steroid secretion to treat infertility and various diseases including endometriosis and prostate cancer.[52]

A unique feature of the GnRHR is that it lacks an intracellular carboxy terminal tail.[53,54] It has been proposed that the active conformation of the receptor is not phosphorylated and resistant to rapid desensitization and internalization as there is no recruitment of β-arrestin.[55] After prolonged treatment with an agonist, the GnRH receptor is down-regulated. This probably occurs by a β-arrestin independent internalization pathway.[52]

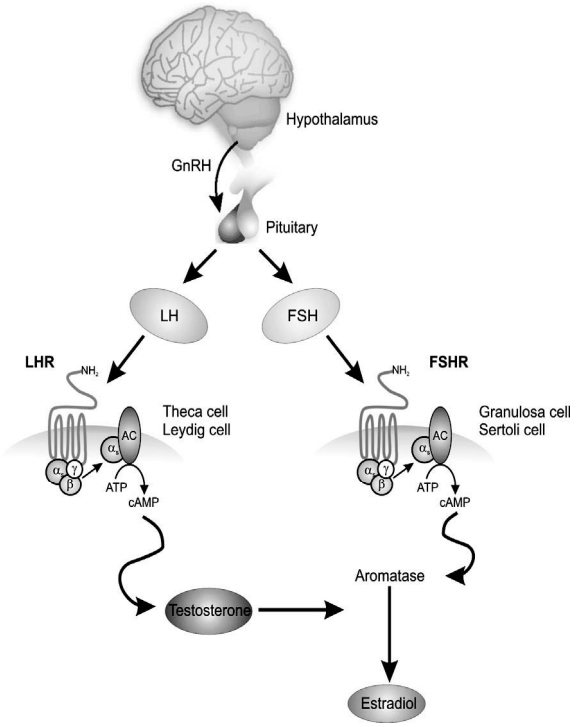
There is some literature evidence that dimerization of the GnRHR plays a role in signal transduction.[56] Already more than two decades ago, Conn *et al* conducted several studies on monomeric and dimeric peptide-based GnRHR modulators.[21] In these studies, peptide

antagonists were modified to allow binding to monoclonal antibodies, resulting in bivalent ligands in which the antibody acts as the spacer. It was observed that ligand dimerization resulted in GnRHR agonism. This was rather unexpected since the monomeric peptides showed an antagonistic effect on GnRHR signaling. In contrast, when the GnRH antagonist was connected to a 12-15 Å spacer it remained an antagonist. From these studies it was concluded that GnRHR dimerization is a prerequisite for biological functioning and that an antagonist becomes an agonist when it is capable of bridging two receptor molecules within a critical distance between 15 and 150 Å. These observations were later corroborated by studies using genetically engineered cells expressing the GnRHR fused to either red fluorescent protein (GnRHR-RFP) or green fluorescent protein (GnRHR-GFP). When treated with an effective agonist, the GnRHR was shown to aggregate, which was evidenced by enhanced red fluorescence that results from fluorescence resonance energy transfer (FRET) from GnRHR-GFP to GnRHR-RFP. Such increase in FRET was not observed when an antagonist was used.[57] Other studies using RET also confirmed agonist-induced dimerization. In this studies the receptor pairs were equipped with a GFP and yellow fluorescent protein (YFP)[58] or with *renilla* luciferase and YFP.[59] To further establish the unique properties of GnRHR and the role of its lacking C-terminal tail, it was investigated whether this aggregation resulted in the localization of the receptor in cellular microdomains that are rich of cholesterol and sphingolipids (also known as lipid-rafts).[60] Other GPCRs such as the muscarinic receptor[61] and the B_2 bradykinin receptor[62] undergo localization to such lipid-rafts under the influence of an agonist. For the GnRHR it has been established that this receptor resides in such lipid-rafts and is not dependent on stimulation of an agonist. Depletion of cholesterol from the membranes resulted in malfunction of the receptor suggesting that these microdomains hold both receptor and G-proteins.[60]

Glycoprotein Hormone Receptors

The glycoprotein hormone receptors (GpHRs)[63,64] are part of a large family of G protein coupled receptors (GPCRs) that are distinguished by the nature of their endogenous ligands, the glycoprotein hormones. Three distinct GpHRs exist in man, namely the luteinizing hormone/choriogonadotropin receptor (LH/CGR),[65] the follicle-stimulating hormone receptor (FSHR)[66] and the thyroid-stimulating hormone receptor (TSHR).[67] The first two are key mediators in the human reproduction system whereas the TSHR controls endocrine production of the thyroid gland. The three GpHRs are highly homologous in their seven transmembrane α-helical part (the domain characteristic for the GPCR superfamily) and diverge in their extracellular domains. Although these domains fall into the so-called large N-terminal leucine rich repeat (LRR) category, they are differentiated such that each GpHR binds specifically and with high affinity to its glycoprotein hormone counterpart.

Box 6: Signaling pathway of receptors in human reproduction



Pulsatile GnRH secretion from the hypothalamus is regulated by the GPR54 receptor. After binding of GnRH to its receptor in the pituitary, the synthesis and release of the gonadotropins LH and FSH is regulated. These hormones in turn bind to their receptors in the gonads and thus regulate the production of hormones such as testosterone and estradiol (via enzymatic conversion by aromatase). These hormones in turn inhibit the formation of GnRH from the hypothalamus providing a negative feedback loop to regulate hormone levels.

There are four glycoprotein hormones described to date, each composed of an identical α-subunit and a unique β-subunit. Luteinizing hormone (LH) and human chorionic gonadotropin (hCG) both bind and agonize the LHR, follicle-stimulating hormone (FSH) activates the FSHR and thyroid-stimulating hormone (TSH) induces TSHR signaling. For all receptor/hormone pairs, the combination of the unique β-subunit in the glycoprotein in combination with the nature of the LRR domain of the receptor is at the basis of selective ligand/receptor binding.

Activation of the LHR and the FSHR results in the formation of cAMP via G_s proteins.[63] Stimulation of phopholipase C and subsequent inositol phosphate formation is also involved in GpHR signaling. However, there are some contrary results whether this is a consequence of G_i or G_q activation. In females, activation of the receptors by FSH and LH stimulates germ cell maturation and estradiol and progesterone production in the ovaries. In males, Leydig cells in the testes are stimulated to produce testosterone. The hormones estradiol and progesterone in turn inhibit the secretion of GnRH from the hypothalamus allowing a negative feedback loop to regulate hormone levels.

There is some compelling literature evidence detailing that GpHRs exert their activity in dimerized form. Dimers of each of the three GpHRs have been observed to exist on the cell surface as evidenced by resonance energy transfer experiments.[68] For the LHR, some researches reported receptor self-association in the absence of an agonist[69] while others only observed receptor aggregation when an agonist was added.[70,71] Contrary, for the TSHR it was observed that receptor oligomers dissociate in the presence of an agonist.[72] Research by Roess and co-workers showed that functional LH receptors migrate to form aggregates that are localized in lipid-rafts.

In addition, as was observed for the GnRHR,[60] disruption of the lipid-rafts also resulted in impaired signaling for wild-type LHR.[73] The signaling of a constitutively active LHR mutant was not impaired when the lipid-rafts were disrupted, suggesting that establishing and maintaining receptor interactions is of more importance in signaling than the membrane microenvironment.[74]
Extensive research by Ji and co-workers showed that a mutant GpHR can *trans*-activate a unliganded receptor by its large N-terminal domain.[75] In this studies, one receptor lacking the ligand binding domain and the other lacking the G protein binding domain proved to function properly when coexpressed. More surprisingly, it was shown that such *trans*-activation resulted in the generation of either the cAMP signal or the inositol phosphate signal for the FSHR, but not both.[76] This evidences that receptor dimerization and/or cross-activation are involved in the specificity of GpHR signaling.
Recent research by Costagliola and co-workers showed that GpHRs can form homodimers and heterodimers via interactions of the transmembrane domain and that this dimerization is associated with a strong negative cooperativity upon ligand binding.[68] Negative cooperativity has been described as a way to respond over a wide range of agonist concentrations, with maximal sensitivity obtained in the lower concentration range.[77] It was suggested that negative cooperativity could play an important role in defining the characteristics for GpHR signaling.
Direct evidence of GpHR dimerization was obtained by the X-ray structure in which the FSHR ecto-domain was organized in a dimeric fashion (Figure 4).[78] The interaction of the FSH ecto-domains was determined to be only weak, but is suggested to be stronger when the receptor is located in its native membrane-bound state. It is not possible to speculate on the transmembrane organization from the crystal structure. However, it has been proposed that close transmembrane contacts are possible.[79] In addition, it is estimated that close transmembrane interaction results in the binding of one FSH hormone to a receptor dimer, as depicted in Figure 4. This phenomenon is in agreement with the negative cooperativity findings by Costagliola and co-workers.[68]

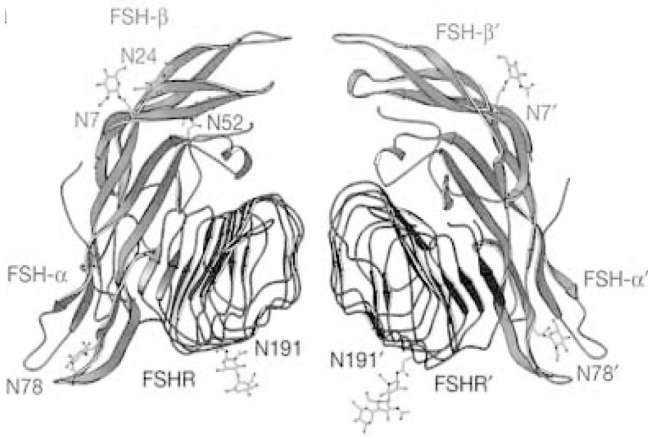
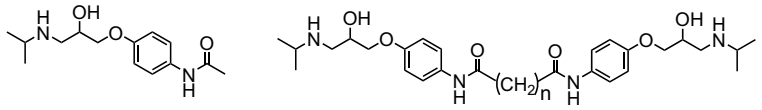


Figure 4. Ribbon diagram of bound FSH to the truncated FSH receptor (FSHR) showing the dimerization of two of these complexes. Figure adapted from reference 78.

Bivalent ligands

Since the appreciation that signaling may be guided for specific GPCRs by receptor homo- or heterdimerization, several reports have appeared describing the design of dimeric ligands that target specific GPCRs. Sometimes these bivalent ligands exhibit increased potency and selectivity when compared to their monovalent counterparts.[80-82]

Dimeric ligands **2**, derived from the β-adrenergic antagonist practolol **1**, is one of the earliest examples of a dimeric ligand described in literature.[83] Compounds of general formula **2** showed up to 160–fold increased binding affinities for the β-adrenergic receptor when compared to monomer **1**. In addition, the selectivity of the dimeric compounds for two subtype receptors (that is, the β_1/β_2-adrenergic receptors) seems to be dependent on the number of atoms between the two ligands.

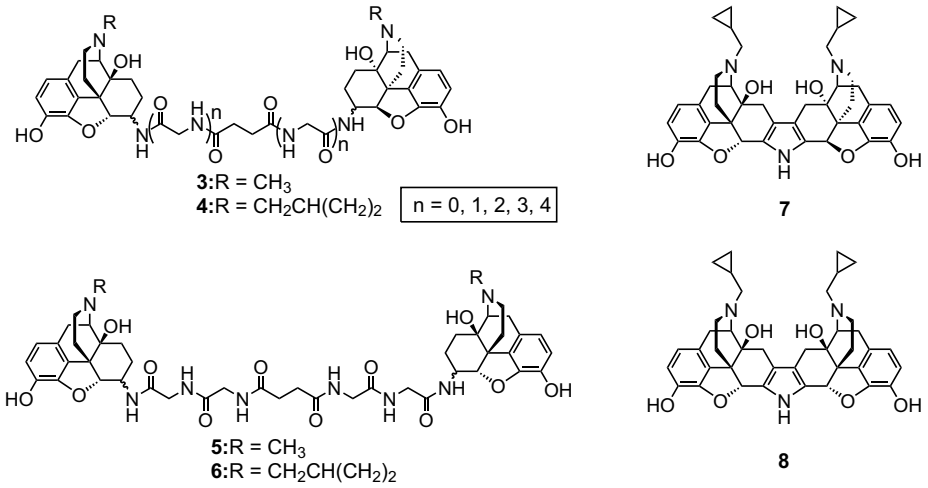


1: Practolol **2**

Major pioneering work in the field of receptor modulation by dimeric ligands was directed to the opioid receptors.[84] Three subtypes of opioid receptors have been identified by pharmacological and biological studies, namely the μ-, κ- and δ-opioid receptors. The bivalent ligand method proved to be useful for the development of more potent and selective modulators. Portoghese and co-workers reported a maximum increase of the potency on the μ-opioid receptor for dimeric ligands **3** (derived from two agonists) and **4** (derived from two antagonists), with a spacer length of 22 atoms between the two ligands (n=2).[85-87] When the binding properties of the dimeric ligands **3** were evaluated a clear trend was observed, correlating the binding affinity with the agonistic potency. Remarkably, for dimeric ligands **4**, no such correlation between the binding affinities and antagonistic potencies was found. In this case, all dimeric ligands had similar affinities for the receptor, independent of the spacer length. This effect was ascribed to a strong negative cooperativity of **4** with the receptor dimer (Box 7). In order to investigate whether both ligands bind two separate receptor dimers, additional compounds were prepared in which one of the pharmacophores was replaced by the inactive enantiomer (distomer).[88] Both compounds **5** and **6** were significantly less potent compared to **3** and **4** with the same spacer length. It was therefore concluded that the dimeric ligands **3** and **4** bridge two separate but identical neighboring opioid recognition sites.

A second remarkable observation was made from the dimeric ligand series **4**, having an antagonistic potency on both the μ- and the κ-opioid receptor. The potency on the receptors strongly depended on the used spacer length and the compound with the shortest spacer (n=0) proved to be the most potent and selective κ-opioid ligand from this series. As a consequence, compound **7** was prepared, incorporating the shortest 'spacer' possible and this compound proved to be a highly potent and selective κ-opioid antagonist.[89] The mesomeric compound **8** in which

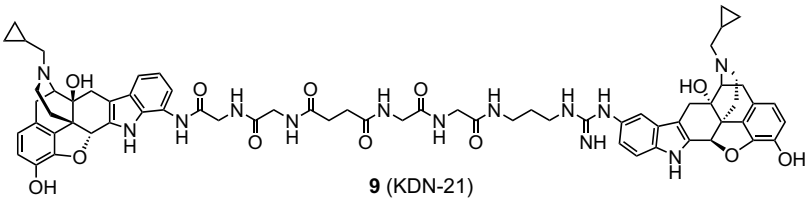
the second half of the ligand can not be recognized by an identical binding site was selective for the κ-opioid receptor and slightly more potent than **7**. This rather surprising result was attributed to the fact that not the pharmacophore itself, but a specific part of the molecule was responsible for the selectivity observed for the κ-opioid receptor.[90] Later, it appeared that the ring-nitrogen in the second ligand was responsible for the selectivity for the κ-opioid receptor and that the second ligand serves as a scaffold facilitating the right orientation of the amine moiety to interact with specific sites that are unique for the κ-opioid receptor.



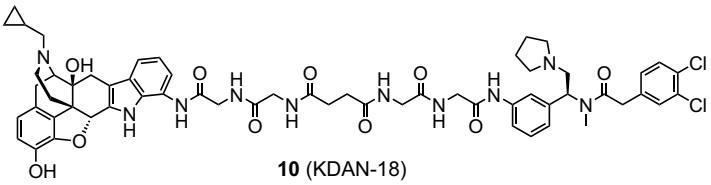
Box 7: Model for negative cooperativity in the interaction of a homodimeric antagonist with neighboring receptor sites of a GPCR dimer. Receptor binding of one recognition unit of the dimeric ligand induces a conformational change in the neighboring binding site of the receptor dimer. A rapid switch between the univalently bound sites would lead to the apparent blockage of both sites. Figure adapted from reference 84.

More recent research by Portoghese and co-workers led to the development of dimeric ligands that specifically activate and bridge heterodimeric κ- and δ-opioid receptors. Immunoprecipitation studies by Jordan and Devi showed that κ- and δ-opioid receptors form heterodimers.[39] Binding affinities of compound **9**, that consists of a κ-opioid selective antagonist on one side and a δ-opioid selective antagonist on the other, are 200-fold higher for cells that co-express both κ- and δ-opioid receptors than for cells in which the κ- and δ-opioid are expressed independently and mixed.[91] The compounds with shorter or longer spacers had less affinity for the receptor dimer. The fact that the monomeric selective κ-opioid ligand could antagonize a selective δ-opioid agonist, was reason to hypothesize that dimeric compound **9** binds to a heterodimeric receptor that is allosterically coupled (and thus able to influence ligand binding on the other receptor) rather than bridging two receptor homodimers that are not allosterically

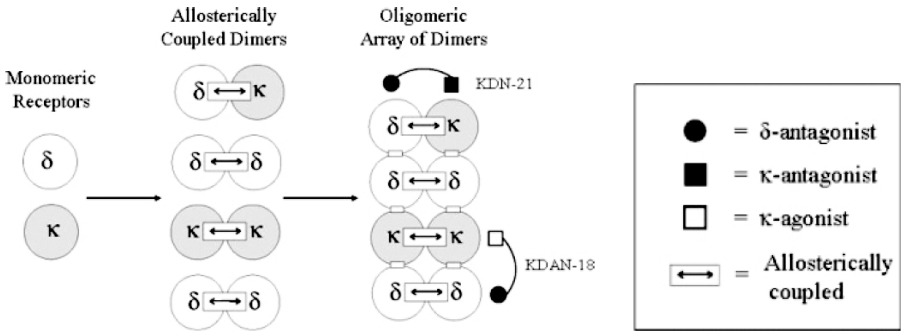
coupled (as depicted below). This theory was further supported by investigating the properties of a set of heterodimers **10** that were derived from a selective κ-opioid antagonist on one side and a selective δ-opioid agonist on the other.[92] It is proposed that, due to cooperativity between the receptors, allosterically coupled receptor dimers occur in either an agonist state or an antagonist state. Dimeric ligands with an agonist on one side and an antagonist on the other would then preferentially bind to two neighboring δ-δ and κ-κ homodimeric receptors instead of binding to a δ-κ heterodimer. The binding affinity of compound **10** was 65-fold higher for cells that co-expressed both receptors than for mixed cells that expressed one specific receptor. Furthermore, no allosteric effect was observed for compound **10** after adding monomeric antagonists. Also, compound **10** could not be antagonized by compound **9**, which further supports the assumption that **10** binds to a different receptor dimer than **9**, as depicted below.



9 (KDN-21)

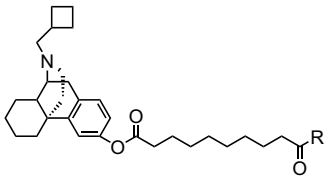


10 (KDAN-18)

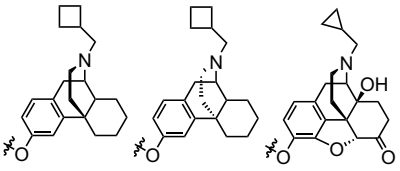


Neumeyer and co-workers independently reported on dimeric ligands **15-17** that target opioid receptors. Butorphan **11** is a potent but non-selective κ- and μ-opioid receptor agonist. The homodimer of **11** with a 10-carbon ester spacer (that is, compound **15**) proved to be the most potent compound in the series which shows a two-fold improved binding affinity for the κ- and μ-opioid receptors compared to **11**.[93] Notably, monomeric compound **14** has a reduced affinity for the receptors compared to **11**, which was attributed to the side chain that hampered binding of the ligand to the receptor sites.[94] For dimeric ligand **16**, that consists of one active- and one less active enantiomer on either side of the spacer, reduced receptor affinities were observed.[95] Based on this observation and the fact that the dimeric ligand **15** has (slightly) higher affinity for the receptor than the two monomeric pharmacophores **14**, the authors concluded that the dimeric ligand **15**

should bridge two opioid receptors. An interesting activity profile is observed for heterodimeric compound **17**, that consists of a non-selective κ- and μ-opioid receptor agonist on one side and the potent κ-opioid antagonist **13** on the other. Here, no antagonistic properties were observed and the compound proved to be a full agonist for the κ-opioid receptor. One may conclude that receptor dimerization or oligomerization is involved here since antagonistic activities are expected when the receptors are uncoupled in a monomeric fashion.[94]

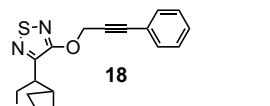


14: R = OH
15: R = 11
16: R = 12
17: R = 13



11 **12** **13**

The muscarinic receptor family consists of 5 subtypes ($M_{1\text{-}5}$) receptors and attempts to develop selective modulators for one of these were only moderately successful during the years. Recently, two groups reported independently on the development of more selective agonists for the muscarinic subtypes. Compounds **18**, **19** and **20** have high affinity for all muscarinic receptor subtypes.[96] Some binding selectivity was observed for M_1 and M_2 with compound **20**, which proved to be a partial agonist for all receptor subtypes with slight selectivity for M_1 and M_4. Compound **19** showed only partial agonistic activity on M_1 and M_4 and no activity on M_3 and M_5. In comparison, monomeric ligand **18** is a potent agonist for all subtype receptors. Similar results were obtained when more flexible spacers were used. Here, the optimal spacer length for dimeric ligands with high affinity and potency proved to be 11 atoms as in compound **22**.[97] Also, an improved selectivity for the M_1 and M_4 receptors was observed. The selectivity could be further improved using a different pharmacophore on one side as present in compounds **23** and **24**. Varying the spacer length in these compounds influenced the binding affinity and selectivity for the receptor subtypes. For example, compound **23** showed the highest binding affinity on M_4, while compound **24** had most affinity for M_2.[98]



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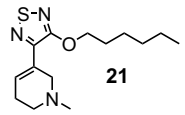

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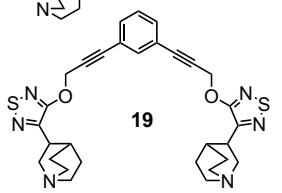

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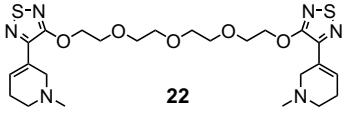

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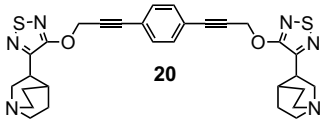

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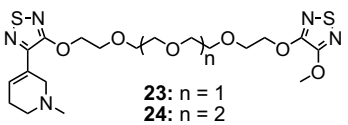
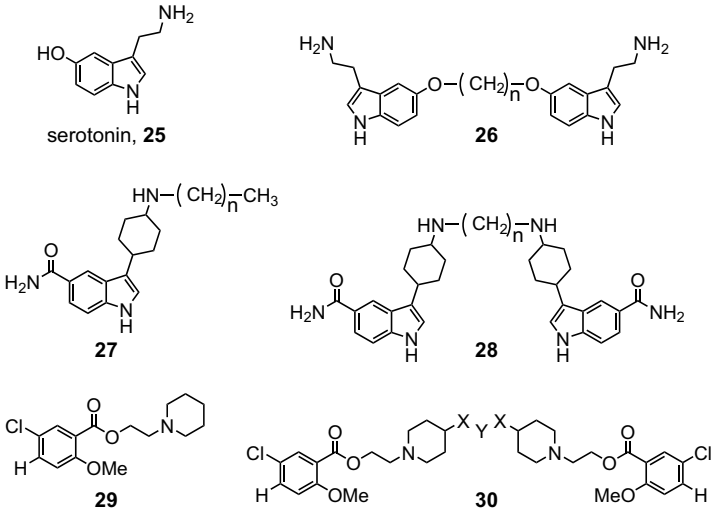


23: n = 1
24: n = 2

To date, seven classes of serotonin (5-HT) receptors have been reported and, with the exception of 5-HT_3, all are G-protein coupled. Class 1 is further divided in 5 subtypes (5-HT_{1A-F}) and class 2 in 3 subtypes (5-HT_{2A-C}). In order to develop more selective ligands for the 5-HT_1 receptor, Halazy and co-workers reported on dimers of serotonin (5-HT, **25**) with varying spacers. Compound **26** has a higher affinity and potency on the 5-$HT_{1b/d}$ than on the 5-HT_{1a} receptor when compared to serotonin.[99] This phenomenon was independent of the nature and the length of the spacer. Others reported the interesting observation that dimeric ligands **28** (derived from agonist **27**) with a spacer of 7 or 8 methylene units possessed high affinity and selectivity for 5-$HT_{1b/d}$ in comparison with the 5-HT_{1a} receptor. Moreover, a reverse trend was observed for compounds with shorter or longer spacer length, that were more selective for the 5-HT_{1a} receptor.[100] Based on these results it has been postulated that the increased affinity observed for dimeric serotonin ligands was attributed to both pharmacophores in the molecule while the selectivity for the 5-$HT_{1b/d}$ receptor was a result of the positioning of the spacer to serotonin.



More recent research on the serotonin receptor was based on the specific 5-HT_4 partial agonist **29**. Berque-Bestel and co-workers reported on dimeric ligands **30** that contain flexible alkyl or ethylene glycol spacers and compounds with more rigid aromatic spacers.[101,102] For most compounds it appeared that the binding affinities of the dimers were in the same order of magnitude as monomeric ligand **29**. Interestingly, depending on the nature, the length and the attachment mode of the spacer, the activity profile changed for some compounds. For example, compound **30** (in which X = NHCO and Y = $(CH_2)_{10}$) was a potent antagonist while compounds with shorter spacer lengths (Y = 5, 3 or 2 methylene units) or other modes of attachments (X = CONH, NHCO) still possessed some agonistic properties. For a set of dimeric ligands based on a aromatic scaffold, the agonistic activity was also reduced compared to compound **29**.[101] To evaluate whether the dimeric ligands truly interact with a receptor dimer, additional RET studies were performed. The compounds were added to cells that express 5-HT_4 receptors equipped with both a *renilla* luciferase tag and a yellow fluorescent protein and for some of these compounds an increase in BRET signal was observed. This effect was more pronounced for compounds that

contain longer spacers (more then 20-24 atoms, corresponding to approximately 22 Å distance between the two ligands). Compounds with short spacer lengths or very rigid scaffolds did not show an increase in BRET signal.[102]

From the examples described above, it is clear that targeting receptors with dimeric ligands is a useful method to study receptor dimerization or oligomerization in more detail. In some examples, ligand dimerization led to an increase in potency and/or selectivity for subtype receptors or a change in the activity profile of the parent compound. Several hypotheses have been postulated to rationalize the improved pharmacological profiles observed, including the following (represented in Figure 5); A) the bivalent ligand interacts simultaneously with two neighboring receptors, B) the bivalent ligand occupies both its primary binding site and a secondary, low affinity binding site on the same receptor protein located in close proximity to the primary site, C) enhanced binding affinity proceeds through a univalently bound state, the unbound recognition unit being in the locus of neighboring binding sites (this would be equivalent to a high local concentration of the free pharmacophore) and D) the bivalent ligand induces or stabilizes receptor dimerization.[16,84]

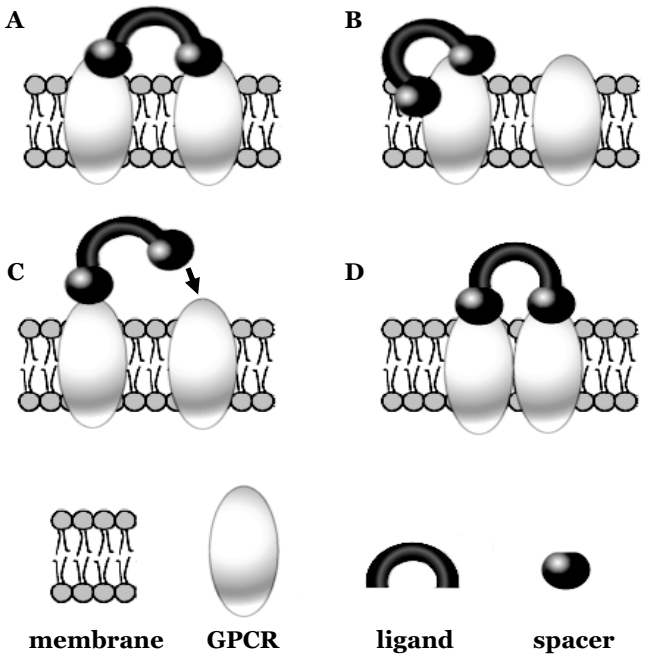


Figure 5. Schematic representation of bivalent ligand binding. A) Binding at neighboring GPCRs. B) Binding at secondary binding site. C) Increased local concentration of free pharmacophore. D) Induction/stabilization of GPCR dimerization.

Which of these mechanisms is involved in binding of dimeric ligands to GPCRs is not well established in most cases and limits the development of dimeric ligands with predictable pharmacological profiles.[81] It is evident that successful binding of a dimeric ligand to a receptor (dimer) highly depends on the spatial orientation of the pharmacophore and the spacer used to connect the two ligands. Obviously, the thermodynamics of the binding process of the dimers to

the receptors are decisive.[103] Productive binding requires a favorable free energy change (ΔG = negative), that is dependent on enthalpic (ΔH) and entropic (ΔS) components (and temperature). Different scenarios can be considered. For instance, the binding of one of the ligands in a dimer may facilitate the binding of the second ligand to the same receptor (positive cooperativity) resulting in a more negative ΔH. In reverse, binding of the first ligand may disrupt binding of the second ligand, resulting in an enthalpically diminished binding (negative cooperativity). The entropic change for dimeric ligands is greatly influenced by the linker between the ligands. Major loss in entropy (unfavorable) may occur when the translational, rotational or conformational freedom is reduced upon binding. The use of rigid linkers between two ligands diminishes the loss in entropy compared to the use of flexible linkers. However, on the other hand, rigid linkers also reduce the probability for a dimeric ligand to bind a receptor dimer because of its restricted spatial orientation. There is no general rule for the successful development of dimeric ligands with enhanced pharmacological properties and therefore a systematic approach in the design and synthesis of dimeric ligands is needed. Consequently, the research described in this thesis is based on a series of combinatorial chemistry approaches to maximize the diversity in ligands, ligand attachment sites, spacer rigidity, spacer length and hydrogen-bond forming potential.

Outline of the thesis

One of the major scientific challenges in the study of GPCR dimerization is the lack of reliable (bio)structural information. Although receptor homology models can be built based on the bovine rhodopsin and recently reported β-adrenergic X-ray structures,[33-35] the spatial alignment of a membrane-anchored receptor dimer can not be rationalized. In order to study the phenomenon of GPCR dimerization in more detail a ligand-based approach, in which two distinct pharmacophores are connected by a spacer system of variable length and rigidity, emerges as an appealing option. The research described in this thesis is based on the design, synthesis and pharmacological evaluation of dimeric ligands that target specific GPCRs that are involved in human reproduction. Up to date, there is no literature precedent of dimeric ligands derived from low molecular weight pharmacophores that is focused on this class of GPCRs.

The first part of the thesis describes an approach to prepare a set of dimeric ligands that target the gonadotropin-releasing hormone receptor (GnRHR). With the exception of the antibody-ligated dimeric peptide agonists described in the introduction, no precedents on dimeric GnRHR ligands have appeared to date. It has been estimated from the crystal structure of rhodopsin that the distance between two ligand binding sites is approximately 31 Å when the rhodopsin dimers are contacted at transmembrane helices 4 and 5. Recent literature reports on potent dimeric ligands for opioid receptors and serotonin receptors support the notion that the distance between two pharmacophores should be around 22 Å. Molecular modeling studies that are based on the GnRHR homology model showed that two GnRHR ligands that are interconnected by 23-atom spacer is sufficient to bridge two separate receptors (Figure 6).

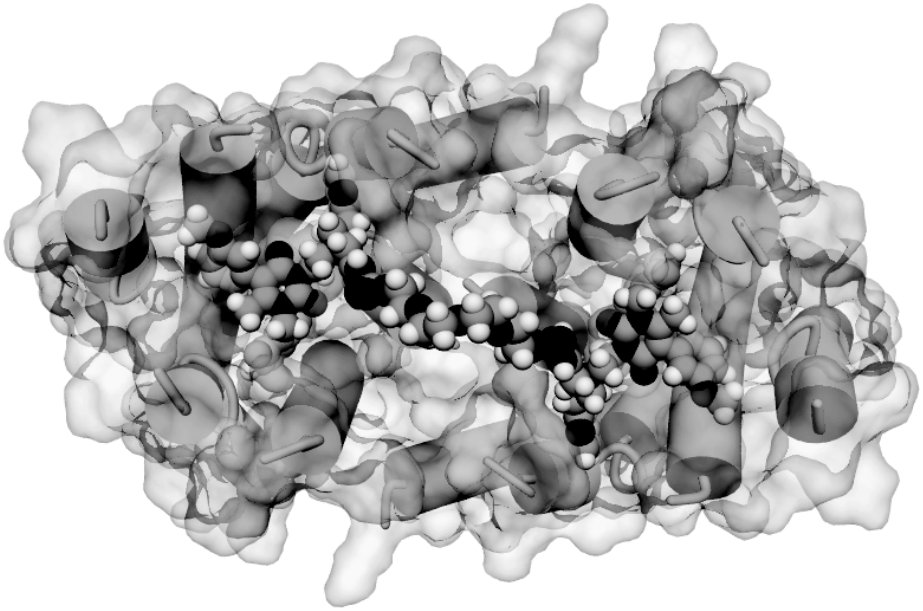
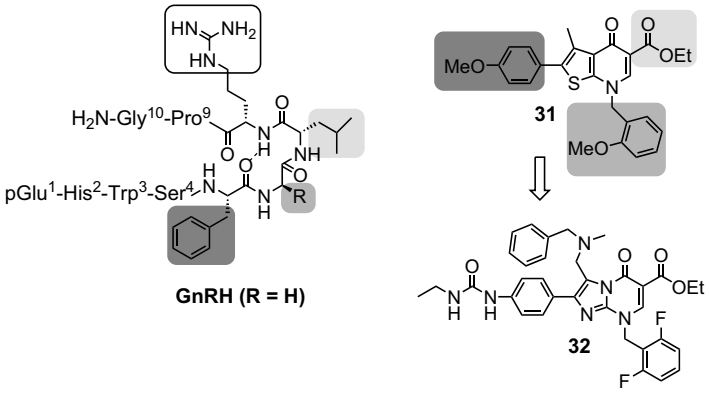


Figure 6. Molecular modeling of a GnRHR dimer containing two ligands that are interconnected by a 23-atom spacer molecule.

Several types of molecules have been reported as GnRHR modulators, covering peptide-based agonists and antagonists and non-peptidic antagonists. Screening the inhibitory effects of compounds on the human GnRHR led to the discovery of compound **31** that exhibits 67% inhibition at 20 μM. Comparison of **31** with GnRH revealed a correlation in the structural orientation of the aromatic and lipophilic side chains on the heterocyclic scaffold. Further optimization of the lead structure led to the discovery of a set of GnRHR antagonists bearing a tertiary amine (such as present as in compound **32**) that most likely interacts with the receptor residues that originally bind Arg^8 in GnRH.



Chapter 2 describes a strategy towards dimeric ligands that is flexible both with respect to the nature and size of the linker entity and to the site to which the linker is attached to the monovalent ligand. Screening of the available literature information on the structure-activity relationships around **32** revealed three positions within ligand **32** that may be amenable for functionalization without completely compromising antagonistic activity. These are the tertiary, benzylic amine (as long as the pK_a is not largely affected), the urea moiety (of which the ethyl functionality may be substituted) and the ethyl ester position. Differently functionalized ligands were connected to spacers that were flexible and hydrophilic in character. The synthesis and pharmacological evaluation of all compounds on the GnRHR, including a set of monomeric reference compounds, is presented.
The influence of the linker moiety between the two pharmacophores in a dimeric construct may contribute significantly to the bioactivity of dimeric ligands. **Chapter 3** describes the synthesis and pharmacological evaluation of dimeric ligands that contain a more rigid linker system. The linkers were derived from a benzene core that is substituted on various positions with an acetylene function.
Recent reports on the mode of binding of various GnRHR antagonists detailed that different residues are involved in binding of different classes of antagonists. It was further reported that some molecules are insurmountable antagonists for the receptor. Insurmountable antagonists generally have a significantly long residence time on the receptor hampering the design of successful dimeric ligands. **Chapter 4** describes the synthesis of dimeric GnRHR antagonists that are based on a different core structure than used in the previous chapters.

The second part of this thesis focuses on the development of dimeric ligands for the glycoprotein hormone receptors (GpHRs), namely the luteinizing hormone receptor (LHR) and the follicle-stimulating hormone receptor (FSHR). As described in the introduction, GpHRs normally bind a ~30 kDa large glycoprotein hormone. From a therapeutic point of view, the ability to influence (agonize or antagonize) a specific GPCR with a small molecule having pharmacologically favorable properties is a major research objective. In endogenous events revolving around human reproduction both LHR and FSHR are often simultaneously activated, and selective therapeutic control in stimulation of one of these receptors is currently only possible with the aid of their unique glycoprotein ligands. Most effective small molecule GpHR modulators described to date are thought to exert their biological activity by binding to the seven-helical transmembrane region. Molecular pharmacology studies making use of specifically mutated LHRs in combination with signal transduction experiments unambiguously point towards an allosteric binding site for these small molecule agonists, which most likely is located within the LHR transmembrane region. **Chapter 5** describes the development of two series of dimeric ligands that were derived from a previously reported low molecular weight LHR agonist. The parent ligand also shows activity on the FSHR, albeit with a lower potency. The bivalent ligand approach was used to investigate whether an increase in selectivity or potency could be obtained for one of the receptors.

Some of the linker molecules delineated in Chapters 3 and 5 are characterized by a proline motif. This motif was recognized as a scaffold molecule that can be obtained by a novel Staudinger aza-Wittig Ugi multi component reaction (SAWU-MCR). The SAWU-MCR was previously used on carbohydrate derived azido-aldehydes to obtain oligo-hydroxyl substituted proline derivatives. **Chapter 6** describes the use of this MCR to obtain dimeric ligands that are rigid in nature, but due to the multiple hydroxyl functions are expected to include improved solubility properties and increasing hydrogen-bond forming potential.

Chapter 7 explains the use of an oligoproline (OP) linker for the development of dimeric ligands. It has been established that oligoprolines adopt a helical conformation. It was investigated whether OPs may be functionalized with the LHR agonist used in Chapters 5 and 6 without disturbing its helical conformation. It was envisioned that the rigid OP helix still possesses sufficient flexibility for a precise interaction of the dimeric ligand with the receptors. In addition, some compounds were prepared and pharmacologically evaluated that incorporate more than two ligands.

Although some of the dimeric compounds discussed in Chapters 2-7 have bioactivity properties that differ from their monomeric counterparts, it is difficult to establish whether the ligands truly bind to two distinct receptor molecules. **Chapter 8** describes the use of a FSHR pharmacophore that incorporates a stereocenter in the molecule. It was previously established that one enantiomer is a potent antagonist while the other is inactive on the FSH receptor. The synthesis of dimeric ligands that consist of only the active enantiomers (eutomers) and dimeric ligands bearing both enantiomers (eutomers and distomers) may provide different activity profiles that help in the elucidation of the interaction mode of dimeric ligands with GpHRs.

Since the LHR agonist described in Chapters 5-7 are also active on the FSHR, this ligand can also be regarded as FSHR agonist. Interesting compounds may arise when such an agonist is combined with an FSHR antagonist. **Chapter 9** deals with the synthesis and biological evaluation of such 'hetero'-dimeric compounds. To further explore the binding mode of these dimers, mixtures of the individual ligands were also pharmacologically evaluated.

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Chapter 2

Synthesis and evaluation of homodimeric GnRHR ligands[1]

Introduction

The gonadotropin-releasing hormone receptor (GnRHR) is a well-studied GPCR, with the decapeptidic gonadotropin-releasing hormone (GnRH) acting as its endogenous ligand.[2, 3] GnRH is secreted by the hypothalamus and operates as a key regulator in mammalian sexual maturation and reproductive functions. After binding of GnRH to the GnRHR, the release of gonadotropins (LH and FSH) in anterior pituitary gonadotropes is stimulated. The major signal transduction route for the GnRHR is via the $G\alpha_q$ protein which induces release of intracellular calcium. Several types of molecules have been reported as GnRHR modulators, covering peptide-based agonists and antagonists and non-peptidic antagonists.[4-6] Imidazopyrimidinone **1**[7-9] (Figure 1) is a well-documented example of a low molecular weight antagonist with high affinity for the GnRHR. Interestingly, up to this date, no non-peptidic agonist for the GnRHR has been reported.

There is some literature evidence that dimerization of the GnRHR plays a role in signal transduction (see for a more detailed description, Chapter 1).[10-15] Already more than two decades ago, several studies on monomeric and dimeric peptide-based GnRHR modulators were

conducted.[11] In these studies, peptide antagonists were modified to allow binding to monoclonal antibodies, resulting in bivalent ligands in which the antibody acts as the spacer. It was observed that ligand dimerization resulted in GnRHR agonism, which was rather unexpected since the monomeric peptides showed an antagonistic effect on GnRHR signaling. From these studies it was concluded that GnRHR dimerization is a prerequisite for biological functioning. These observations were later corroborated by studies using genetically engineered cells expressing the GnRHR fused to both red fluorescent protein (GnRHR-RFP) and green fluorescent protein (GnRHR-GFP). When treated with an effective agonist, the GnRHR was shown to aggregate, which was evidenced by enhanced red fluorescence that results from fluorescence resonance energy transfer (FRET) from GnRHR-GFP to GnRHR-RFP.[13] Other studies using RET also confirmed agonist-induced dimerization. In this studies the receptor pairs were equipped with a GFP and yellow fluorescent protein (YFP)[14] or with *renilla* luciferase and YFP.[15]

One of the major scientific challenges in the study of GnRHR dimerization, as with all GPCRs, is the lack of reliable (bio)structural information. Although GnRHR homology models can be built based on the bovine rhodopsin X-ray structure, the spatial alignment of a membrane-anchored GnRHR dimer can not be rationalized. In order to study the phenomenon of GnRHR dimerization in more detail a ligand-based approach, in which two distinct GnRHR pharmacophores are connected by a spacer system of variable length and rigidity, emerges as an appealing option. In the context of a research program aimed at understanding and modulating GnRHR mediated signaling, it was decided to design a set of well defined dimeric ligands based on the earlier reported[7] and potent antagonist **1**. With the exception of the antibody dimerized peptide agonists described above, no precedents on dimeric GnRHR ligands had appeared at the outset of the here presented study. It was decided to focus on a strategy towards dimeric ligands that is flexible both with respect to the nature and size of the linker entity and to the site to which the linker is attached to the monomeric ligand.

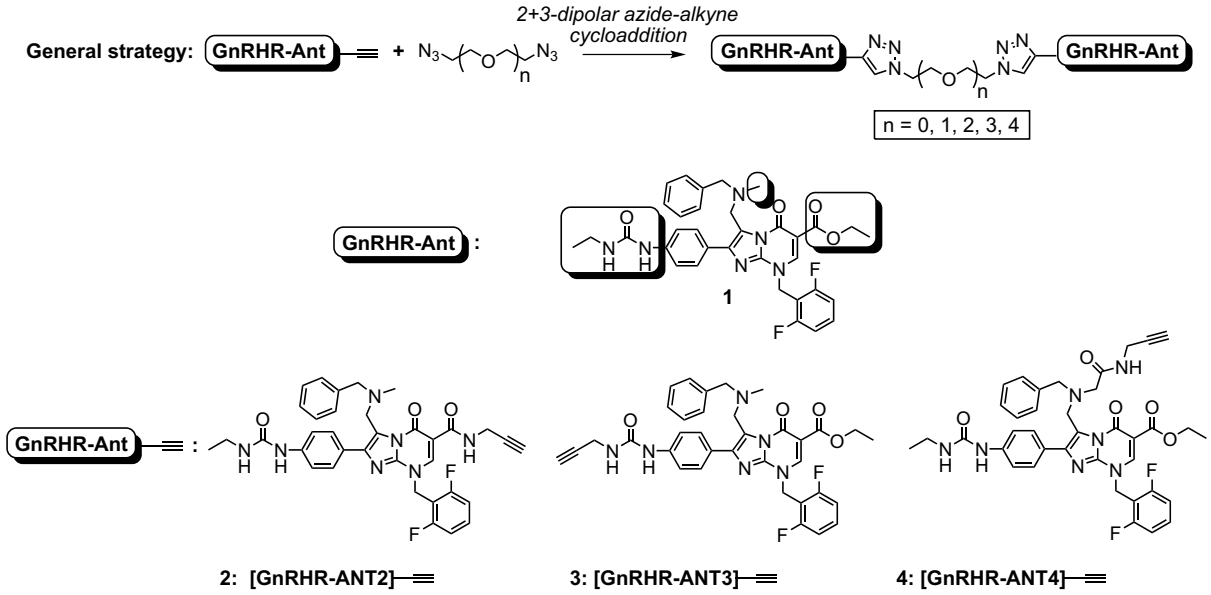
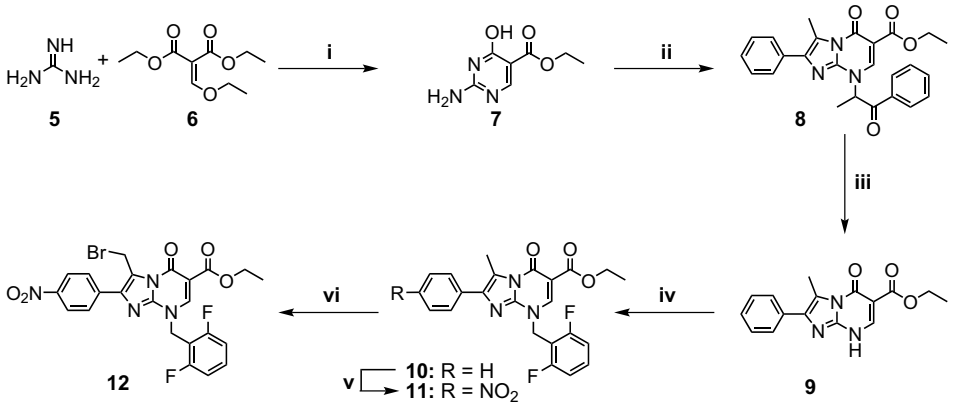


Figure 1. Design of the compound library.

Results and Discussion

The design of the compound library is outlined in Figure 1 and involves the introduction of a terminal acetylene onto the imidazopyrimidinone ligand **1**, followed by homo-dimerization to a bis-functionalized azide spacer by means of the copper-catalyzed 1, 3-dipolar Huisgen cycloaddition now commonly referred as the Click reaction.[16, 17] As spacer entities a set of ethylene glycols were selected differing in length and equipped with an azide at both termini for coupling to two acetylene containing ligand moieties. Screening of the literature information[7] on the structure-activity relationships around **1** has revealed three positions within ligand **1** that may be amenable for functionalization without completely compromising antagonistic activity. These are the tertiary, benzylic amine (as long as the pK_a is not largely affected), the urea moiety (of which the ethyl functionality may be substituted) and the ethyl ester position. Based on this information, it was decided to synthesize acetylene derivatives **2**, **3** and **4**, and couple these to the bisazides through the Click reaction. In order to evaluate a potential effect of bivalency, the corresponding monomeric ligand-spacer derivatives were also prepared. This chapter describes the synthesis of the dimeric ligand library. Further, the propensity of the compound library to bind to the GnRHR, relative to lead compound **1**, and to antagonise GnRH-mediated GnRHR signaling, is presented.

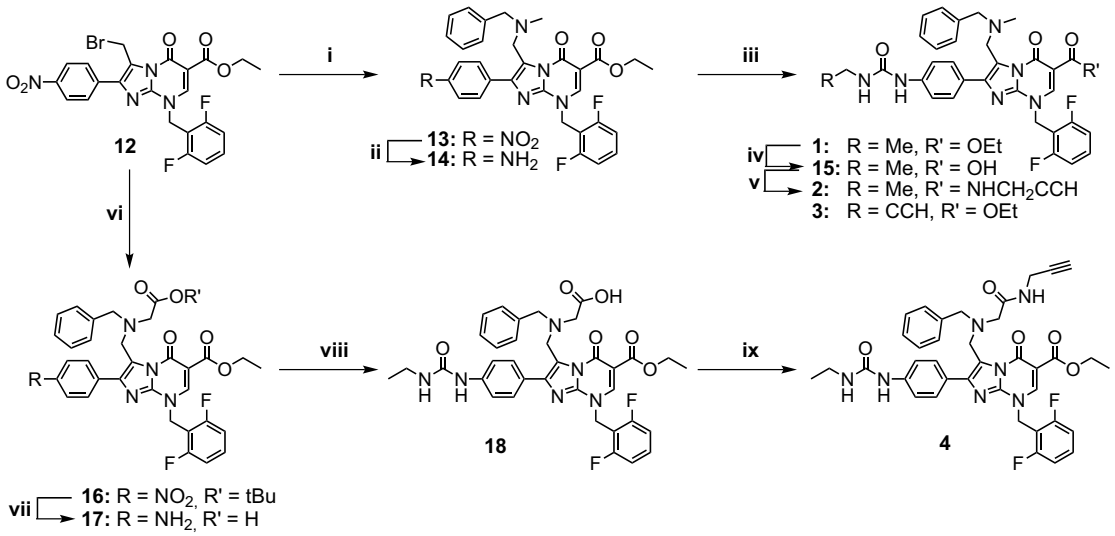
The synthesis of acetylene derivatives **2**, **3** and **4** is based on the common key intermediate **12** (Scheme 1), which is in fact an advanced intermediate in the literature synthesis of ligand **1**.[7-9] Following this procedure, but with some adaptations, compound **12** was readily prepared as follows (Scheme 1). Guanidine **5** and diethyl 2-(ethoxymethylene)malonate **6** were condensed to form pyrimidine **7** in 79% yield. Reaction of **7** with α-bromopropiophenone gave a mixture of *O*- and *N*-alkylated products of which **8**, the major regioisomer, was isolated after crystallization in 39% yield. Cleavage of the phenacyl moiety in **8** with zinc in acetic acid gave phenylimidazopyrimidinone **9**. Subsequent alkylation with 2, 6-difluorobenzyl bromide and subsequent aromatic substitution with sodium nitrate in concentrated sulfuric acid provided compound **11** which was further brominated to obtain key intermediate **12**.



Scheme 1. Synthesis of the intermediate building block **12**. *Reagents and conditions*: *i*. NaOEt, EtOH, rt, 4 d, 79%; *ii*. α-bromopropiophenone, KI, K_2CO_3, DMF, rt, 5 d, 39%; *iii*. Zn, HOAc, 80 °C, 6 h, 88%; *iv*. 2, 6-difluorobenzyl bromide, KI, K_2CO_3, DMF, rt, 18 h, 70%; *v*. $NaNO_3$, conc. H_2SO_4, 0 °C, 4 h, 82%; *vi*. NBS, AIBN, CCl_4, 80°C, 2 h, 83%.

Compound **12** was then aminated with *N*-methyl-*N*-benzylamine after which the nitro functionality in **13** was reduced to the amine using iron in an acidic ethanol solution (Scheme 2). Subjecting the obtained amine **14** to ethyl isocyanate in pyridine resulted in GnRHR antagonist **1**. Saponification of the ethyl ester in **1** and subsequent coupling with propargyl amine under standard peptide condensation conditions gave acetylene derivative **2** in 63% yield over the two steps. Acetylene functionalized ligand **3** was obtained in one step from amine **14** by reaction with propargyl isocyanate, which was freshly prepared from propargyl amine and diphosgene (71% yield).

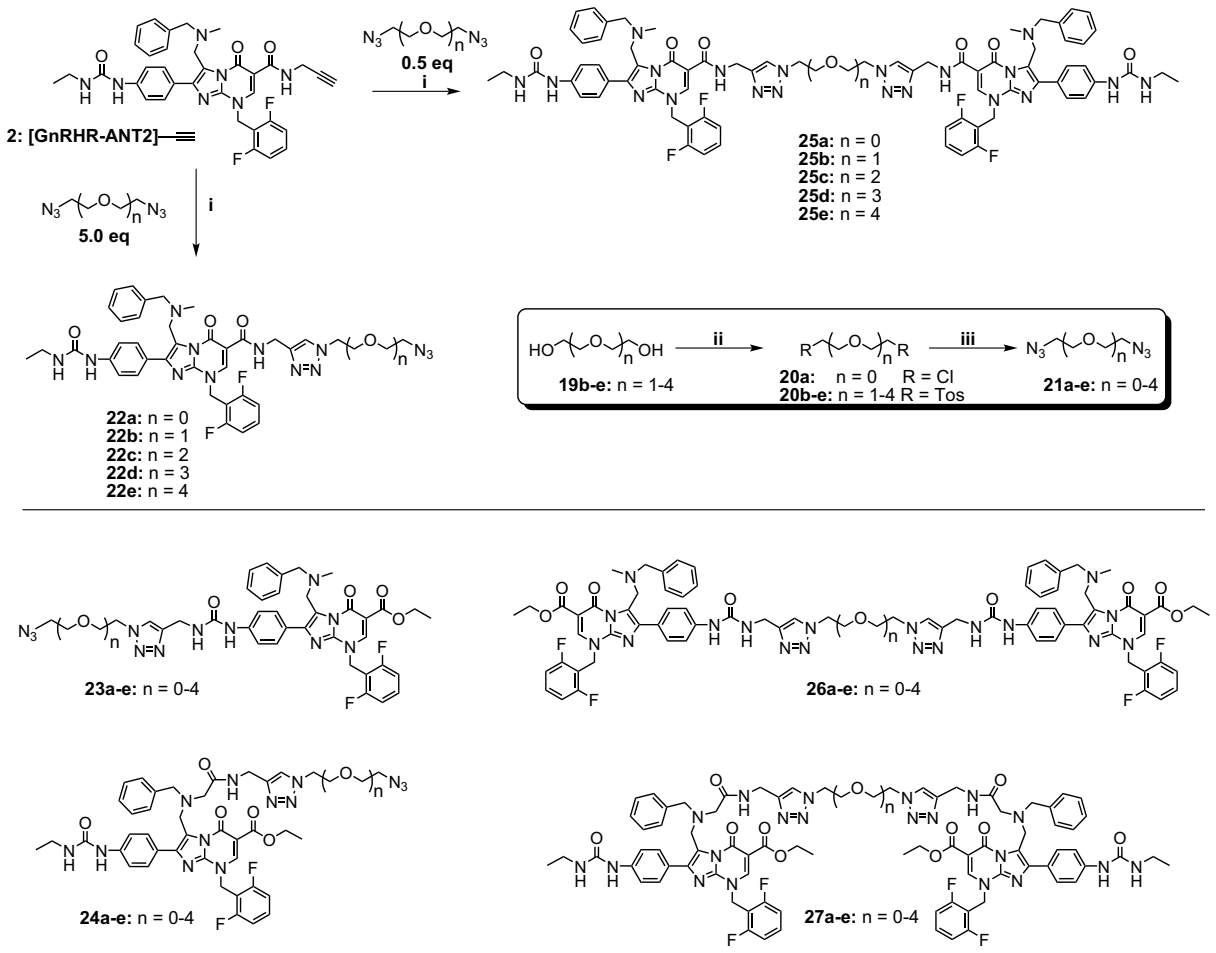
The synthesis of **4** was accomplished as follows. Nucleophilic displacement of the allylic bromide in **12** by *tert*-butyl 2-(benzylamino)acetate (DiPEA, THF) gave tertiary amine **16** almost quantitatively. At this stage, reduction of the nitro functionality using the conditions previously applied for the transformation of **13** to **14** (iron, acidic ethanol) proved abortive, since next to producing the desired aniline also transesterification of the *tert*-butyl ester to the corresponding ethyl ester was observed. Switching to the iron/dichloromethane/trifluoroacetic acid reagent system however gave simultaneous reduction of the nitrophenyl to the aniline and acidolysis of the *tert*-butyl ester, to produce zwitterionic compound **18** in excellent yield. Treatment of **18** with ethyl isocyanate in pyridine, followed by condensation of the carboxylic acid with propargylamine (BOP, DiPEA, DMF) gave target compound **4** in 38% over the two steps.



Scheme 2. Synthesis of acetylene ligands. *Reagents and conditions*: *i*. *N*-methyl-*N*-benzylamine, DiPEA, THF, rt, 18 h, 97%; *ii*. Fe, conc HCl, EtOH, rt, 4 h, 85%; *iii*. ethyl isocyanate, pyridine, rt, 18 h, 88% (R = Me) *or* propargylamine, diphosgene, EtOAc, reflux, 4 h, then **14** in pyridine, rt, 18 h, 71% (R = CCH); *iv*. 1M LiOH, H_2O/MeOH/THF (12/2/1; v/v/v), rt, 1 h, 83%; *v*. propargylamine, BOP, DiPEA, DMF, rt, 18 h, 75%; *vi*. *tert*-butyl 2-(benzylamino)acetate, DiPEA, THF, rt, 18 h, 97%; *vii*. Fe, TFA/DCM (1/1; v/v), rt, 18 h, 97%; *viii*. ethyl isocyanate, pyridine, rt, 18 h, 71%; *ix*. propargylamine, BOP, DiPEA, DMF, rt, 18 h, 54%.

Having the acetylene functionalized ligands **2**, **3** and **4** in hand, the synthesis of bisazides **21a-e** and the envisaged Click reaction to the target dimeric structures was performed. The required bisazide **21a** was prepared from dichloroethane as reported and stored in solution.[18] The

bisazides **21b-e** were prepared from the corresponding ethyleneglycol derivatives **19b-e** by 1) transformation to bis(paratoluenesulfonate) esters **20b-e** and 2) displacement of the sulfonate esters by azide ion. The synthesis of the dimeric ligands and their monomeric ligand-spacer counterparts is presented in Scheme 3. As an example, acetylene **2** is reacted with each of the 5 bisazides **21a-e**, with n = 0, 1, 2, 3 and 4, under the agency of a catalytic amount of copper sulfate and sodium ascorbate in a mixture of *tert*-butanol, acetonitrile and water. Employing a five-fold excess of the bisazide **21a-e** showed complete conversion of **2** (RP-HPLC) and afforded the monosubstituted derivatives **22a-e** as their TFA salt in yields ranging from 30 to 47 % after preparative HPLC purification. The dimeric ligands were obtained by applying essentially the same conditions, but with 0.5 equivalents of bisazides **21a-e**, giving target compounds **25a-e** (13 to 36%). In the same way, and with comparable efficiency, monosubstituted compounds **23a-e** and **24a-e**, as well as dimeric compounds **26a-e** and **27a-e** were prepared.



Scheme 3. Representative route of the synthesis of monomeric- and dimeric GnRHR ligands. *Reagents and conditions*: *i*. $CuSO_4$, sodium ascorbate, *t*BuOH/CH_3CN/H_2O (2/1/1; v/v/v), 60 °C, 3 h; *ii*. KOH, TsCl, DCM, 0 °C, 3 h, >95%; *iii*. NaN_3, TBAI, DMF, 80 °C, 18 h, >95%.

Subsequently, the dimeric ligands **25a-e**, **26a-e** and **27a-e** were tested on their ability to bind to the GnRH receptor and to antagonize its stimulation by GnRH. As reference compounds, known antagonist **1**, the three acetylene derivatives **2**, **3** and **4** and the mono-functionalized azides **22a-e**, **23a-e** and **24a-e** were included in the assays. The results are summarized in Table 1. For the functional assay, Chinese hamster ovary (CHO) cells were used, stably transfected with the GnRHR and equipped with the calcium sensitive NFAT luciferase reporter gene. To measure antagonistic efficacy, the GnRHR cells were stimulated with a submaximal (EC_{80}) concentration of the agonist in the presence of several concentrations of the test compounds. Antagonistic activity is detected as a decrease of the luminescence signal upon addition of the luciferase substrate. The IC_{50} values of the compounds in the antagonistic assay are listed in column A of Table 1.

The binding affinity of the compounds was monitored in a displacement assay on membrane fractions of GnRHR-expressing CHO cells with the radioactive GnRHR agonist [^{125}I]triptorelin. At first hand, single-point assay for all compounds was performed, in which the percentage of [^{125}I]triptorelin displacement at 1 μM compound was determined (Column B). Furthermore, for a small number of ligands, including the reference compounds and a few dimeric ones, K_i values were determined (Column C). Introducing bivalency might affect the intrinsic activity of the compounds and thus change their profile from an antagonist into an agonist. Additional assays performed with all compounds in an agonistic set-up, that is, when tested alone in the luciferase reporter gene assay, did not provide any actives (data not shown).

The combined results of both antagonistic and radioligand assays indicate that lead compound **1** exhibits more antagonistic potency than all newly synthesized compounds. IC_{50} values (column A in Table 1) are always lower than the available corresponding K_i values (column C). This is not surprising since a high concentration of GnRH is also present in the functional assay. Interestingly, monomeric analogues **3** and **4** show a comparable binding affinity (K_i) as ligand **1**, but they are less active in the functional assay. Replacement of the ethyl ester in **1** with a propargyl amide, as in compound **2**, strongly reduces antagonistic potency. None of the compounds incorporating this modification showed a significant antagonistic effect in our functional assay at concentrations up to 3 μM. They do, however, displace radioligand binding to some extent at a concentration of 1 μM (Table 1, column B). The results of the monomeric and dimeric ligands based on acetylene derivatives **3** and **4** have higher affinities. In the series based on **3** the >10 fold increase in antagonistic potency going from monomeric ligand **23d** to dimeric ligand **26d** is noteworthy. The series based on acetylene derivative **4** suggests that the monomeric ligands are slightly more active than their dimeric counterparts. However, the difference in this series is too marginal to allow interpretation with respect to potential bivalent modes of binding.

		A	B	C			A	B	C
	n	IC_{50} (nM) [a]	% displ. at 1 µM [b]	K_i (nM) [b]		n	IC_{50} (nM) [a]	% displ. at 1 µM [b]	K_i (nM) [b]
		Monomeric ligands					***Dimeric ligands***		
1		76	99	5					
2	-	>3000	85	162					
22a	0	>3000	78	n.d.	**25a**	0	>3000	26	n.d.
22b	1	>3000	68	n.d.	**25b**	1	>3000	64	n.d.
22c	2	>3000	81	n.d.	**25c**	2	>3000	73	n.d.
22d	3	>3000	69	n.d.	**25d**	3	>3000	86	n.d.
22e	4	>3000	70	n.d.	**25e**	4	>3000	85	n.d.
3	-	724	98	1					
23a	0	>3000	76	n.d.	**26a**	0	>3000	68	n.d.
23b	1	>3000	85	n.d.	**26b**	1	>3000	88	n.d.
23c	2	>3000	84	n.d.	**26c**	2	1006	84	23
23d	3	>3000	84	n.d.	**26d**	3	362	90	33
23e	4	668	90	n.d.	**26e**	4	1534	89	n.d.
4	-	1134	98	5					
24a	0	719	74	n.d.	**27a**	0	2215	65	n.d.
24b	1	535	66	n.d.	**27b**	1	1781	71	n.d.
24c	2	472	83	n.d.	**27c**	2	849	84	59
24d	3	735	76	n.d.	**27d**	3	1540	71	69
24e	4	832	78	n.d.	**27e**	4	311	89	195

Table 1. Functional and binding properties to GnRHR for the mono- and dimeric ligands. The mean IC_{50} are calculated from the –log IC_{50} values from two or three independent experiments performed in duplicate. The SD of pIC_{50} is generally lower than 0.3. [a] CHO cells that stably express the GnRHR were stimulated with a submaximal (EC_{80}) concentration of GnRH and were incubated with increasing concentrations of the compounds. The IC_{50} value is the concentration of compound needed to inhibit the agonistic response by 50%. [b] Membranes of GnRHR-expressing CHO cells were incubated with the radioactive GnRHR agonist [^{125}I]triptorelin. The percentage of [^{125}I]triptorelin displacement by incubating the fractions with a single concentration of 1 µM of test compound (Column B); K_i values were determined (Column C) by incubating the membranes with increasing concentrations of test compound. The K_i value is calculated based on the concentration of compound needed to displace 50% of radioligand. The mean K_i are calculated from the -log K_i values from three independent experiments performed in duplicate. The SD of pK_i is generally lower than 0.2. [^{125}I]triptorelin. displ. = displacement. n.d. = not determined.

Conclusion

To establish the role of GnRHR dimerization on signaling, the scope of dimeric ligands needs to be broadened. Changes in the attachment sites of the spacer and variations in the spacer moiety itself should provide more structure-activity information. Moreover, also hetero-dimeric ligands, incorporating two distinct GnRHR pharmacophores, are required. The synthetic strategy presented here, allowing sequential introduction of two ligands, seems to be suitable for the preparation of libraries with broader diversity, provided that the corresponding acetylene-modified ligands and azide-containing spacers of the desired nature are available. Furthermore,

additional (bio)chemical experiments concerning compounds with significantly enhanced potency compared to their monomeric counterpart, such as **26d**, is vital in order to elaborate on the potential modes of binding to GnRHR as delineated in Figure 1. To address these questions in more detail, the influence of the nature of the linker system on the bioactivity of the dimeric ligands was investigated and described in Chapter 3.

Experimental Procedures

GnRHR Luciferase reporter gene assay

Chinese Hamster Ovary, CHO-K1, cells with stable expression of the human Gonadotropin-Releasing Hormone Receptor (GnRHR) and Nuclear Factor Activated T-cell luciferase reporter gene were grown to 80-90% confluence in culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% w/v Fetal Bovine Serum, 100 units/mL Penicillin and 100 µg/mL Streptomycin and 400 µg/mL Geniticin. On the day of the assay, cells were washed twice with Phosphate Buffered Saline and then harvested with cell dissociation solution. Cells were resuspended in assay medium consisting of DMEM supplemented with 1 mg/L insulin and 5 mg/L apo-transferrin and 3% v/v DMSO. Then, 10 µL cell suspension containing 7,500 cells was added to each well of a 384-well white culture plate. Thereafter, 10 µL of test compound was added at 10 concentrations ranging from final concentration of 10 µM to 0.3 nM with half log intervals. Compounds were allowed to preincubate with cells for 30 min followed by addition of 10 µL agonist GnRH at a final concentration of 3 nM which produces approximately 80% of the maximal effect (EC_{80}) when given alone. After 4 h stimulation, 15 µL of luclite® was added to each well for detection of luciferase protein and plates were left at room temperature for 1 h in the dark. Finally, the luminescence signal was quantified on the TopCount® Microplate Scintillation and Luminescence Counter.

Radioligand Binding Assay.

Ganirelix was provided by Schering-Plough research institute (Oss, The Netherlands). [^{125}I]triptorelin (specific activity 2200 Ci mmol^{-1}) was purchased from Perkin Elmer Life Sciences B.V. (Groningen, The Netherlands). CHO-K1 cells stably expressing the human GnRH receptor were provided by Schering-Plough research institute (Oss, The Netherlands). All other chemicals and cell culture materials were obtained from standard commercial sources.

CHO (Chinese hamster ovary) -K1 cells expressing the wild-type human GnRH receptor were grown in Ham's F12 medium containing 10% bovine calf serum, streptomycin (100 µg mL^{-1}), penicillin (100 IU mL^{-1}) and G418 (0.4 mg mL^{-1}) at 37 °C in 5% CO_2. The cells were subcultured twice weekly at a ratio of 1:20. For membrane preparation the cells were subcultured 1:10 and transferred to large 14-cm diameter plates. For membrane preparation the cells were detached from the plates by scraping them into 5 mL PBS, collected and centrifuged at 1400 *g* (3000 rpm) for 5 min. Pellets derived from 30 plates were pooled and resuspended in 30 mL of ice-cold 50 mM Tris-HCl buffer supplemented with 2 mM $MgCl_2$, pH 7.4. An UltraThurrax was used to homogenize the cell suspension. Membranes and the cytosolic fraction were separated by centrifugation at 100,000 *g* (31,000 rpm) at 4 °C for 20 min. The pellet was resuspended in 10 mL of the Tris-HCl buffer and the homogenization and centrifugation steps were repeated. Tris-HCl buffer (10 mL) was used to resuspend the pellet and the membranes were stored in 500 µL aliquots at -80 °C. Membrane protein concentrations were measured using the BCA (bicinchoninic acid) method.[19]

On the day of the assay membrane aliquots containing 20 μg protein were incubated in a total volume of 100 μL assay buffer (50 mM Tris HCl, pH 7.4, supplemented with 2 mM $MgCl_2$ and 0.1% BSA) at 22 °C for 45 min. Displacement experiments were performed using five concentrations of competing ligand in the presence of 30,000 cpm [^{125}I]triptorelin. Non-specific binding was determined in the presence of 1 μM ganirelix and represented approximately 20% of the total binding. Total binding was determined in the presence of buffer and was set at 100% in all experiments, whereas non-specific binding was set at 0%. Incubations were terminated by dilution with ice-cold Tris HCl buffer. Separation of bound from free radioligand was performed by rapid filtration through Whatman GF/B filters pre-soaked with 0.25 % PEI for 1 h using a Brandel harvester. Filters were subsequently washed three times with ice-cold wash buffer (50 mM Tris HCl, pH 7.4, supplemented with 2 mM $MgCl_2$ and 0.05% BSA). Filter-bound radioactivity was determined in a γ-counter.

All data was analyzed using the non-linear regression curve-fitting program GraphPad Prism v. 4 (GraphPad Software Inc, San Diego, CA, U.S.A.). Inhibitory binding constants (K_i values) were derived from the IC_{50} values according to $K_i = IC_{50}/(1 + [C]/K_d)$ where [C] is the concentration of the radioligand and K_d its dissociation constant.[20] The K_d value (0.35 nM) of [^{125}I]Triptorelin was obtained by computer analysis of saturation curves.[21] All values obtained are means of at least three independent experiments performed in duplicate.

Chemical procedures.
Reactions were executed at ambient temperatures unless stated otherwise. All moisture sensitive reactions were performed under an argon atmosphere. All solvents were removed by evaporation under reduced pressure. Reactions were monitored by TLC analysis using silica gel coated plates (0.2 mm thickness) and detection by 254 nm UV-light or by either spraying with a solution of $(NH_4)_6Mo_7O_{24} \times 4H_2O$ (25 g/L) or $(NH_4)_4Ce(SO_4)_4 \times 2H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at ~150 °C. Column chromatography was performed on silica gel (40-63 μm). NMR spectra were recorded on a 200/50 MHz, 300/75 MHz, 400/100 MHz, 500/125 MHz or 600/150 MHz spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants (*J*) are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. For LC-MS analysis, a HPLC-system (detection simultaneously at 214 and 254 nm) equipped with an analytical C_{18} column (4.6 mmD × 250 mmL, 5μ particle size) in combination with buffers A: H_2O, B: CH_3CN and C: 1% aq. TFA and coupled to a mass instrument with an electronspray interface (ESI) was used. For RP-HPLC purifications, an automated HPLC system equipped with a preperative C_{18} column (5 μm C_{18}, 10Å, 150 × 21.2 mm) was used. The applied buffers were A: H_2O + 0.1% TFA and B: CH_3CN. High resolution mass spectra were recorded by direct injection (2 μL of a 2 μM solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60000 at *m/z* 400 (mass range *m/z* = 150-2000) and dioctylpthalate (*m/z* = 391.28428) as a lock mass. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan).

Ethyl 2-amino-4-hydroxypyrimidine-5-carboxylate (7). To a solution of guanidine carbonate (18.0 g, 200 mmol) in ethanol (300 mL) were added sodium ethoxide (13.6 g, 200 mmol) and diethyl ethoxymethylene malonate (40.0 mL, 200 mmol). After stirring for 4 d the volatiles were removed, the residue dissolved in water (500 mL) and neutralized with aqueous HCl (2M). The titled compound was collected by filtration as a white powder (29.0 g, 158 mmol, 79%). ESI-MS *m/z*: 183.9 $[M + H]^+$, 206.0 $[M + Na]^+$. ^{1}H NMR (400 MHz, DMSO-*d*6) δ 8.23 (s, 1H), 4.11 (q, 2H, *J* = 7.2), 1.21 (t, 3H, *J* = 6.8). ^{13}C NMR (100 MHz, DMSO-*d*6) δ 163.9 (C), 163.0 (CH), 158.7, 158.2, 103.6 (3 × C), 58.9 (CH_2), 13.8 (CH_3).

Ethyl 5, 8-dihydro-3-methyl-5-oxo-8-(1-oxo-1-phenylpropan-2-yl)-2-phenylimidazo[1, 2-a] pyrimidine-6-carboxylate (8). To a solution of ethyl ester **7** (15.0 g, 81.9 mmol) in DMF (280 mL) were added potassium carbonate (28.3 g, 204.8 mmol), potassium iodide (13.6 g, 81.9 mmol) and 2-bromopropiophenone (30.6 mL, 204.8 mmol). After stirring for 5d the volatiles were removed, the residue dissolved in $CHCl_3$ (500 mL) and washed with water (3 × 200 mL). The organic phase was dried (Na_2SO_4) and concentrated. Flash column chromatography (EtOAc) of the residue afforded crystals which were recrystallized from EtOAc and petroleum light ether. Yield 136.0 g (31.7 mmol, 39%). TLC R_f = 0.7 (5% MeOH in DCM). ESI-MS *m/z*: 430.1 $[M + H]^+$, 452.1 $[M + Na]^+$. ^1H NMR (200 MHz, $CDCl_3$) δ 8.54 (s, 1H), 8.14 (d, 2H, *J* = 8.6), 7.71 – 7.50 (m, 5H), 7.45 – 7.28 (m, 3H), 6.83 (q, 1H, *J* = 7.3), 4.42 (q, 2H, *J* = 7.3), 2.89 (s, 3H), 1.82 (d, 3H, *J* = 7.3), 1.21 (t, 3H, *J* = 6.8). ^{13}C NMR (50 MHz, $CDCl_3$) δ 195.6, 164.2, 156.6 (3 × C), 144.5 (CH), 141.0, 137.6 (2 × C), 134.4 (CH), 133.8, 133.3, 129.0, 128.7, 128.3, 128.0, 127.4 (11 × CH), 122.0, 101.2 (2 × C), 61.1 (CH_2), 57.0 (CH), 16.5, 14.3, 12.9 (3 × CH_3).

Ethyl 5, 8-dihydro-3-methyl-5-oxo-2-phenylimidazo[1, 2-a]pyrimidine-6-carboxylate (9). To a solution of **8** (5.00 g, 11.6 mmol) in acetic acid (120 mL) was added activated zinc (12.1 g, 185 mmol). The mixture was stirred at 80 °C for 6 h, filtered through Celite and the filtrate concentrated. The residue was crystallized by the addition of water (300 mL). The crystals were collected and recrystallized ($CHCl_3$/Et_2O/petroleum light ether) to yield white powder (3.05 g, 10.3 mmol, 88%). TLC R_f = 0.45 (10% MeOH in DCM). ESI-MS *m/z*: 297.9 $[M + H]^+$, 595.2 $[2M + H]^+$. ^1H NMR (600 MHz, $CDCl_3$/CD_3OD) δ 8.68 (s, 1H), 7.54 – 7.47 (m, 5H), 4.34 (q, 2H, *J* = 7.3), 2.86 (s, 3H), 1.39 (t, 3H, *J* = 6.6). ^{13}C NMR (150 MHz, $CDCl_3$/CD_3OD) δ 165.0, 158.0 (2 × C), 157.3 (CH), 147.3, 128.5, 128.3, 127.9, 127.3 (7 × CH), 118.7, 101.3 (2 × C), 59.7 (CH_2), 13.3, 10.8 (2 × CH_3).

8-(2, 6-Difluorobenzyl)-5, 8-dihydro-3-methyl-5-oxo-2-phenylimidazo[1, 2-a]pyrimidine-6-carboxylic acid ethyl ester (10). To a solution of **9** (6.00 g, 20.2 mmol) in DMF (220 mL) were added potassium carbonate (3.07 g, 22.2 mmol), potassium iodide (1.67 g, 10.0 mmol) and bromo-2, 6-difluorotoluene (4.60 g, 22.2 mmol). The mixture was stirred for 18 h, after which the volatiles were removed. The residue was dissolved in a mixture of EtOAc and water (500 mL, 1/1; v/v). The water layer was extracted with EtOAc (2 × 200 mL) and the combined organic layers were dried (Na_2SO_4) and concentrated. The residue was crystallized from $CHCl_3$ and Et_2O to yield 6.08 g (14.3 mmol, 70%) of off-white crystals. TLC R_f = 0.75 (50% EtOAc in toluene). ESI-MS *m/z*: 424.0 $[M + H]^+$, 847.5 $[2M + H]^+$. ^1H NMR (600 MHz, $CDCl_3$) δ 8.36 (s, 1H), 7.68 (d, 2H, *J* = 7.5), 7.43 (t, 2H, *J* = 7.5), 7.39 – 7.37 (m, 1H), 7.33 (t, 1H, *J* = 7.4), 6.99 (t, 2H, *J* = 7.9), 5.50 (s, 2H), 4.37 (q, 2H, *J* = 7.0), 2.90 (s, 3H), 1.38 (t, 3H, *J* = 7.0). ^{13}C NMR (150 MHz, $CDCl_3$) δ 164.2 (C), 161.7 (dd, 2 × C, J_1 = 260.5, J_2 = 6.0), 156.9 (C), 146.2 (CH), 140.6, 138.0, 133.4 (3 × C), 131.6 (t, 1 × CH, *J* = 10.5), 128.4, 128.0, 127.4 (5 × CH), 121.8 (C), 111.8 (dd, 2 × CH, J_1 = 21.0, J_2 = 4.5), 109.6 (t, 1 × C, *J* = 18.0), 100.9 (C), 61.11, 42.9 (2 × CH_2), 14.3, 12.21 (2 × CH_3).

8-(2, 6-Difluorobenzyl)-5, 8-dihydro-3-methyl-2-(4-nitrophenyl)-5-oxoimidazo[1, 2-a]pyrimidine-6-carboxylic acid ethyl ester (11). To a cooled (0 °C) solution of **10** (2.01 g, 4.74 mmol) in concentrated sulfuric acid (20 mL) was added over a period of 30 minutes sodium nitrate (0.40 g, 4.74 mmol). After 4 h, the reaction mixture was poured on ice (100 mL) and $CHCl_3$ was added (150 mL). The organic layer was separated and the water layer extracted with $CHCl_3$ (3 × 50 mL). The combined organic layers were dried (Na_2SO_4) and concentrated. The residue was crystallized from $CHCl_3$ and Et_2O to yield 1.83 g (3.91 mmol, 82%) of yellow crystals. TLC R_f = 0.8 (50% EtOAc in toluene). ESI-MS *m/z*: 469.2 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 8.45 (s, 1H), 8.32 – 7.85 (m, 4H), 7.48 – 6.95 (m, 3H), 5.51 (s, 2H), 4.38 (q, 2H, *J* = 7.0), 2.96 (s, 3H), 1.39 (t, 3H, *J* = 7.0). ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.7 (C), 161.7 (dd, 2 × C, J_1 = 250.0, J_2 = 7.0), 156.7 (C), 147.0 (CH), 146.6, 141.1, 140.1, 135.9 (4 × C), 131.8 (t, 1 × CH, *J* = 11.0), 128.3 (2 × CH), 124.1 (C), 123.6 (2 × CH), 112.2 (d, 2 × CH, *J* = 15.0), 111.7 (t, 1 × C, *J* = 18.0), 101.3 (C), 61.3, 43.2 (2 × CH_2), 14.3, 12.5 (2 × CH_3).

3-Bromomethyl-8-(2, 6-difluorobenzyl)-5, 8-dihydro-2-(4-nitrophenyl)-5-oxoimidazo[1, 2-a]pyrimidine-6-carboxylic acid ethyl ester (12). To a solution of pyrimidine derivative **11** (4.68 g, 10.0 mmol) in carbon tetrachloride (500 mL) were added *N*-bromosuccinimide (1.96 g, 11.0 mmol) and 2, 2'-azobis(isobutyronitrile) (0.33 g, 2.0 mmol). The reaction mixture was stirred at 80 °C until a clear solution was observed (2-3h). The volatiles were removed and the residue was dissolved in $CHCl_3$ (200 mL). The organic layer was washed with aqueous $NaHCO_3$ (200 mL) and the water layer was extracted with $CHCl_3$ (3 × 100 mL). The combined organic layers were dried (Na_2SO_4) and concentrated. The residue was crystallized from DCM and Et_2O to yield 4.55 g (83%) of brown crystals. TLC R_f = 0.4 (50% EtOAc in toluene). ESI-MS *m/z*: 547.2, 549.1 $[M + H]^+$, 569.1, 570.9 $[M + Na]^+$. ^1H NMR (500 MHz, MeOD/$CDCl_3$) δ 8.53 (s, 1H), 8.36 (d, 2H, *J* = 8.8), 8.05 (d, 2H, *J* = 8.8), 7.52 – 7.35 (m, 1H), 7.02 (t, 2H, *J* = 8.0), 5.54 (s, 2H), 5.30 (s, 2H), 4.40 (q, 2H, *J* = 6.6), 1.39 (t, 3H, *J* = 5.1). ^{13}C NMR (500 MHz, MeOD/$CDCl_3$) δ 163.1 (C), 161.4 (dd, 2 × C, J_1 = 250.0, J_2 = 6.3), 156.0 (C), 147.2 (CH), 147.1, 141.6, 140.1, 138.7 (4 × C), 131.6 (t, 1 × CH, *J* = 10.0), 128.4, 123.5 (4 × CH), 123.2 (C), 112.1 (d, 2 × CH, *J* = 21.3), 109.3 (t, 1 × C, *J* = 18.8), 101.1 (C), 62.4, 61.2, 43.4 (3 × CH_2), 13.8 (CH_3).

8-(2, 6-Difluorobenzyl)-5, 8-dihydro-3-(*N*-methyl-*N*-benzylaminometyl)-2-(4-nitrophenyl)-5-oxoimidazo[1, 2-a]pyrimidine-6-carboxylic acid ethyl ester (13). To a cooled (0 °C) solution of bromide **12** (2.74 g, 5.0 mmol) in THF (50 mL) were added benzylmethylamine (0.77 mL, 6.0 mmol) and DiPEA (8.5 mL, 50 mmol). The reaction mixture was stirred for 18 h at ambient temperature after which the volatiles were removed. The residue was dissolved in $CHCl_3$ (200 mL) and the organic layer washed with aqueous $NaHCO_3$ (10%, 200 mL). The water layer was extracted with $CHCl_3$ (3 × 100 mL) and the combined organic layers dried (Na_2SO_4) and concentrated. The crude product was purified by silica gel column chromatography (20% to 50% EtOAc in toluene) to afford 2.84 g (4.83 mmol, 97%) as an off-white solid. TLC R_f = 0.7 (50% EtOAc in toluene). ESI-MS *m/z*: 588.3 $[M + H]^+$. ^1H NMR (600 MHz, $CDCl_3$) δ 8.50 (s, 1H), 8.32 – 8.27 (m, 4H), 7.43 – 7.38 (m, 1H), 7.26 – 7.21 (m, 5H), 7.02 (t, 2H, *J* = 8.4), 5.52 (s, 2H), 4.45 – 4.37 (m, 4H), 3.69 (s, 2H), 2.72 (s, 3H), 1.40 (t, 3H, *J* = 7.2). ^{13}C NMR (150 MHz, $CDCl_3$) δ 163.8 (C), 161.8 (dd, 2 × C, J_1 = 250.5, J_2 = 6.0), 156.5, 146.9 (2 × C), 146.6 (CH), 141.5, 139.8, 139.2, 139.1 (4 × C), 131.8 (t, 1 × CH, *J* = 10.5), 129.1, 129.0, 128.9, 128.8, 128.1, 126.9 (8 × CH), 126.0 (C), 123.7 (CH), 111.9 (dd, 2 × CH, J_1 = 21.0, J_2 = 4.5), 109.5 (t, 1 × C, *J* = 18.0), 101.6 (C), 61.3 (2 × CH_2), 49.7, 43.6 (2 × CH_2), 41.6, 14.4 (2 × CH_3).

2-(4-Aminophenyl)-8-(2, 6-difluorobenzyl)-5, 8-dihydro-3-(*N*-methyl-*N*-benzylaminometyl)-5-oxoimidazo[1, 2-a]pyrimidine-6-carboxylic acid ethyl ester (14). To a cooled (0 °C) solution of nitro derivative **13** (2.65 g, 4.5 mmol) in EtOH (50 mL) were added iron powder (1.26 g, 22.5 mmol) and hydrochloric acid (37%, 26.3 mL, 315 mmol). After 4 h, aqueous $NaHCO_3$ (10% 300 mL), $CHCl_3$ (300 mL) and Hyflo were added. The mixture was filtered, the organic layer was separated and the water layer extracted with $CHCl_3$ (3 × 200 mL). The combined organic layers were dried (Na_2SO_4) and concentrated. Column chromatography of the residue (10% to 50% EtOAc in toluene) afforded red brown crystals (2.12 g, 3.8 mmol, 85%). TLC R_f = 0.5 (10% MeOH in DCM). ESI-MS *m/z*: 558.1 $[M + H]^+$. ^1H NMR (200 MHz, $CDCl_3$) δ 8.37 (s, 1H), 7.86 (d, 2H, *J* = 8.8), 7.46 – 7.11 (m, 6H), 6.99 (t, 2H, *J* = 8.0), 6.76 (d, 2H, *J* = 8.8), 5.50 (s, 2H), 4.38 (q, 2H, *J* = 7.3), 4.31 (s, 2H), 3.65 (s, 2H), 2.17 (s, 3H), 1.38 (t, 3H, *J* = 7.3). ^{13}C NMR (200 MHz, $CDCl_3$) δ 164.2, (C), 161.6 (d, 2 × C, *J* = 257.8), 156.4, 146.2 (2 × C), 145.4 (CH), 141.8, 141.1, 139.7, 138.9 (4 × C), 131.4 (t, 1 × CH, *J* = 10.6), 129.8, 129.0, 127.9, 126.5 (9 × CH), 123.7, 122.0 (2 × C), 114.9 (CH), 111.9 (t, 1 × CH, *J* = 10.5), 101.4 (C), 61.1, 50.4, 43.1 (4 × CH_2), 41.1, 14.3 (2 × CH_3).

8-(2, 6-Difluorobenzyl)-5, 8-dihydro-2-(4-ethylaminocarbonylaminophenyl)-3-(*N*-methyl-*N*-benzylaminometyl)-5-oxoimidazo[1, 2-a]pyrimidine-6-carboxylic acid ethyl ester (1). To a solution of **14** (0.50 g, 0.89 mmol) in pyridine (16 mL) was added ethyl isocyanate (0.35 mL, 0.45 mmol). After 18 h the volatiles were removed and the residue was dissolved in DCM (50 mL). The solution was washed with aqueous $NaHCO_3$ (10%, 25 mL), dried (Na_2SO_4) and concentrated. The residue was purified by silica gel column chromatography (0% to 15% MeOH in DCM). Recrystallization of the solid (DCM, petroleum light ether) afforded the titled compound as an off-white solid (0.49 g, 0.78 mmol, 88%). TLC R_f = 0.6 (10% MeOH in DCM). ESI-MS *m/z*: 629.5 $[M + H]^+$, 1257.8 $[2M + H]^{2+}$. ^1H NMR (500 MHz, $CDCl_3$) δ 8.49 (s, 1H), 8.18 (s, 1H), 7.91 (d, 2H, *J* = 8.5), 7.49 (d, 2H, *J* = 8.5), 7.42 – 7.36 (m, 1H), 7.23 – 7.10 (m, 5H), 7.00 (t, 2H, *J* = 8.0), 5.90 (s, 1H), 5.50 (s, 2H), 4.36 (q, 2H, *J* = 7.5), 4.30 (s, 2H), 3.62 (s, 2H), 3.30 – 3.24 (m, 2H), 2.15 (s, 3H), 1.38 (t, 3H, *J* = 6.6), 1.16 (t, 3H, *J* = 7.3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.2 (C), 161.5 (dd, 2 × C, J_1 = 250.0, J_2 = 7.5), 156.3, 155.2 (2 × C), 145.5 (CH), 140.7, 140.4, 139.5, 138.9 (4 × C), 130.9 (t, 1 × CH, *J* = 10.0), 128.4, 128.1, 127.2, 126.7, 125.9 (7 × CH), 125.8, 121.7 (2 × C), 117.2 (2 × CH), 111.2 (dd, 2 × CH, J_1 = 20.0, J_2 = 3.8), 109.3 (t, 1 × C, *J* = 18.8), 100.4 (C), 60.4, 60.2, 49.7, 42.1 (4 × CH_2), 40.5 (CH_3), 33.8 (CH_2), 14.9, 13.7 (2 × CH_3). HRMS (*m/z*) calcd for $C_{34}H_{34}N_6O_4F_2 + H^+$: 629.26824, obsd 629.26963.

8-(2, 6-Difluorobenzyl)-5, 8-dihydro-2-(4-ethylaminocarbonylaminophenyl)-3-(*N*-methyl-*N*-benzylaminometyl)-5-oxoimidazo[1, 2-a]pyrimidine-6-carboxylic acid (15). To a solution of ethyl acid **1** (1.11 g, 1.77 mmol) in THF (12 mL) were added MeOH (2 mL), water (1 mL) and aqueous LiOH (1 M, 2.66 mmol, 2.66 mL). After 1 h the reaction was complete and the reaction mixture was neutralized with 1M HCl and the volatiles evaporated. The crude product was purified by column chromatography on silica gel (5/94/1 to 30/65/5 MeOH/DCM/H_2O) to yield an off-white solid (0.88 g, 1.5 mmol, 83%). TLC analysis (20% MeOH in DCM) R_f = 0.10. ESI-MS *m/z*: 601.4 $[M + H]^+$, 1201.6 $[2M + H]^{2+}$. ^1H NMR (200 MHz, $CDCl_3$) δ 8.91 (s, 1H), 7.97 (d, 2H, *J* = 8.8), 7.60 (d, 2H, *J* = 8.5), 7.06 – 7.53 (m, 8H), 5.79 (s, 2H), 4.31 (s, 2H), 3.63 (s, 2H), 3.22 (m, 2H), 2.18 (s, 3H), 1.11 (t, 3H, *J* = 7.3). ^{13}C (100 MHz, DMSO-*d*6) δ 162.6 (C), 160.1 (dd, 2 × C, J_1 = 249.5, J_2 = 6.6), 159.0, 153.7 (2 × C), 146.1 (CH), 140.1, 139.4, 139.0 (4 × C), 129.7 (t, 1 × CH, *J* = 10.1), 127.1, 126.9, 126.4, 126.1, 125.9, 125.0 (7 × CH), 124.4, 123.9 (2 × C), 115.8 (2 × CH), 110.0 (d, 2 × CH, *J* = 24.2), 108.6 (t, 1 × C, *J* = 18.8), 100.4 (C), 59.3, 48.4, 42.4 (3 × CH_2), 39.5 (CH_3), 32.4 (CH_2), 13.8 (CH_3).

8-(2, 6-Difluorobenzyl)-5, 8-dihydro-2-(4-ethylaminocarbonylaminophenyl)-3-(*N*-methyl-*N*-benzylaminometyl)-5-oxoimidazo[1, 2-a]pyrimidine-6-carboxylic acid propargyl ester (2). Derivative **15** (0.60 g, 1.0 mmol) and propargylamine (89 µL, 1.3 mmol) were dissolved in DMF (50 ml). BOP (0.59 g, 1.3 mmol) and DiPEA (0.5 mL, 3.0 mmol) were added and the mixture was allowed to stir for 18h. The volatiles were evaporated and the residue redissolved in a DCM/MeOH mixture (200 ml, 9/1; v/v). The mixture was washed with $NaHSO_3$ (10%, 100 mL), $NaHCO_3$ (10%, 100 mL) and water (100 mL). The organic layer was dried ($MgSO_4$) and evaporated. The crude product was purified by silica gel column chromatography (50% to 100% EtOAc in toluene) to yield an off-white solid (0.48 g, 0.75 mmol, 75%). An analytical pure sample for biological evaluation was prepared by an additional purification on a semi-preparative RP-HPLC system (linear gradient of 5.0 CV; 30 to 45%B). ESI-MS *m/z*: 638.4 $[M + H]^+$. ^1H NMR (500 MHz, DMSO-*d*6) δ 9.07 (s, 1H), 9.04 (s, 1H), 8.86 (s, 1H), 7.83 (d, 2H, *J* = 8.4), 7.44 (d, 2H, *J* = 8.4), 7.20 – 7.13 (m, 8H), 6.15 (t, 1H, *J* = 5.2), 5.67 (s, 2H), 4.23 (s, 2H), 4.15 (s, 1H), 3.57 (s, 2H), 3.14 – 3.10 (m, 3H), 2.10 (s, 3H), 1.06 (t, 3H, *J* = 6.8). ^{13}C NMR (125 MHz, DMSO-*d*6) δ 162.3 (C), 161.1 (dd, 2 × C, J_1 = 251.3, J_2 = 7.5), 159.7, 155.0 (2 × C), 146.7 (CH), 141.0, 140.8, 140.2, 139.1 (4 × C), 131.1 (t, 1 × CH, *J* = 10.6), 128.6, 128.5, 128.1, 127.9, 126.7 (6 × CH), 125.7, 121.0 (2 × C), 117.2 (CH), 111.8 (d, 2 × CH, *J* = 25.0), 110.7 (t, 1 × C, *J* = 17.5), 101.3 (C), 81.1 (CH), 73.0 (C), 60.7, 49.7, 43.6 (3 × CH_2), 41.0 (CH_3), 33.9, 28.2 (2 × CH_2), 15.4 (CH_3). HRMS *m/z* calcd for $C_{35}H_{33}N_7O_3F_2 + H^+$: 638.26857, obsd 638.26710.

8-(2, 6-Difluorobenzyl)-5, 8-dihydro-2-(4-propargylaminocarbonylaminophenyl)-3-(*N*-methyl-*N*-benzylaminomethyl)-5-oxoimidazo[1, 2-a]pyrimidine-6-carboxylic acid ethyl ester (3). Propargyl amine (685 µL, 20 mmol) was added to a cooled (ice) solution of diphosgene (970 mg, 50 mmol) in 50 ml EtOAc. The solution was refluxed for 4 hours and the volatiles evaporated. The residue was redissolved in pyridine (50 mL) and amine **14** (2.19 g, 4.00 mmol) was added. The mixture was stirred overnight and evaporated. The crude solid was taken up in $CHCl_3$ (200 mL) and the mixture was washed with $NaHCO_3$ (3 × 100mL). The organic layer was dried ($MgSO_4$) and evaporated. The crude product was purified by silica gel column chromatography (EtOAc/toluene; 2/1) to yield an off-white solid (1.80 g, 2.80 mmol, 71%). An analytical pure sample for biological evaluation was prepared by an additional purification on a semi-preparative RP-HPLC system (linear gradient of 5.0 CV; 30 to 45% B). ESI-MS *m/z*: 639.4 $[M + H]^+$, 661.3 $[M + Na]^+$. ^{1}H NMR (400 MHz, DMSO-*d*6) δ 8.82 (s, 1H), 8.67 (s, 1H), 7.83 (d, 2H, *J* = 8.0), 7.46 (d, 2H, *J* = 8.4), 7.20 – 7.12 (m, 8H), 6.98 (br s, 1H), 5.60 (s, 2H), 4.26 (q, 2H, *J* = 6.4), 4.21 (s, 2H), 3.90 – 3.89 (s, 2H), 3.55 (s, 2H), 3.09 (s, 1H), 2.07 (s, 3H), 1.33 (t, 3H, *J* = 7.2). ^{13}C NMR (100 MHz, DMSO-*d*6) δ 163.7 (C), 161.4 (dd, 2 × C, J_1 = 248.4, J_2 = 7.7), 155.9, 154.6 (2 × C), 147.9 (CH), 141.0, 139.9, 1139.8, 139.1 (4 × C), 131.2 (t, 1 × CH, *J* = 10.0), 128.5, 128.4, 127.9, 126.6, (7 × CH), 124.2, 121.5 (2 × C), 117.7 (2 × CH), 111.8 (d, 2 × CH, *J* = 22.7), 110.8 (t, 1 × CH, *J* = 18.2), 100.0 (C), 82.0 (CH), 72.7 (C), 60.7, 60.2, 49.6, 43.7 (4 × CH_2), 40.8 (CH_3), 28.7 (CH_2), 14.2 (CH_3). HRMS *m/z* calcd for $C_{35}H_{32}N_6O_4F_2 + H^+$: 639.25259, obsd 639.25051.

8-(2, 6-Difluorobenzyl)-5, 8-dihydro-3-(*N*-methyl-N-[*tert*-butyl-2-(benzylamino)acetate])-2-(4-nitrophenyl)-5-oxoimidazo[1, 2-a]pyrimidine-6-carboxylic acid ethyl ester (16). To a cooled (0 °C) solution of **12** (2.74 g, 5.0 mmol) in THF (50 mL) were added *tert*-butyl 2-(benzylamino)acetate (1.33 g, 6.0 mmol) and DiPEA (8.5 mL, 50 mmol). The reaction mixture was stirred for 18 h at ambient temperature after which the volatiles were removed. The residue was dissolved in $CHCl_3$ (200 mL) and the organic layer washed with aqueous $NaHCO_3$ (10%, 200 mL). The water layer was extracted with $CHCl_3$ (3 × 100 mL) and the combined organic layers were dried (Na_2SO_4) and concentrated. The crude product was purified by silica gel column chromatography (20% to 33% EtOAc in toluene) to afford 3.40 g (4.9 mmol, 97%) as an off-white solid. TLC R_f = 0.8 (50% EtOAc in toluene). ESI-MS *m/z*: 688.4 $[M + H]^+$, 710.6 $[M + Na]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.57 (s, 1H), 8.23 – 8.19 (m, 4H), 7.43 – 6.99 (m, 8H), 5.49 (s, 2H), 4.61 (s, 2H), 4.40 (q, 2H, *J* = 7.3), 3.85 (s, 2H), 3.38 (s, 2H), 1.40 (t, 3H, *J* = 6.9), 1.37 (s, 9H). ^{13}C NMR (400 MHz, $CDCl_3$) δ 170.3, 163.1 (2 × C), 161.4 (dd, 2 × C, J_1 = 250.0, J_2 = 7.0), 156.0, 146.6 (2 × C), 146.5 (CH), 141.4, 139.2, 139.0, 138.5 (4 × C), 131.4 (t, 1 × CH, *J* = 10.0), 129.7, 129.3, 128.4, 127.9, 126.6 (7 × CH), 124.7 (C), 123.2 (2 × CH), 111.5 (d, 2 × CH, *J* = 23.0), 109.5 (t, 1 × C, *J* = 18.0), 101.2, 80.0 (2 × C), 60.8, 57.9, 55.0, 47.2, 43.3 (5 × CH_2), 27.7, 14.0(4 × CH_3). HRMS *m/z* calcd for $C_{36}H_{35}F_2N_5O_7 + H^+$: 688.25773, obsd 688.25557.

2-(*N*-((6-(Ethoxycarbonyl)-8-(2, 6-difluorobenzyl)-2-(4-aminophenyl)-5, 8-dihydro-5-oxoimidazo [1, 2-a]pyrimidin-3-yl)methyl)-*N*-benzylamino)acetic acid (17). Iron powder (1.26 g, 22.5 mmol) was added to a cooled (0°C) solution of **16** (3.09 g, 4.50 mmol) in DCM/TFA (150 mL, 1/1; v/v). After stirring for 18h at ambient temperature, the solution was evaporated. Aqueous $NaHCO_3$ (10%, 300 mL), $CHCl_3$ (300 mL) and Celite were added. The mixture was filtered, the organic layer dispensed and the water layer extracted with $CHCl_3$ (3 × 200 mL). The combined organic layers were dried (Na_2SO_4) and concentrated. Column chromatography of the residue (0% to 50% MeOH in EtOAc) afforded red brown crystals (2.61 g, 4.30 mmol, 97%). TLC R_f = 0.2 (10% MeOH in DCM). ESI-MS *m/z*: 602.5 $[M + H]^+$, 1203.3 $[2M + H]^+$. ^{1}H NMR (200 MHz, MeOD/TFA) δ 8.86 (s, 1H), 7.84 (d, 2H, *J* = 8.8), 6.58 (d, 2H, *J* = 8.0), 7.52 – 7.04 (m, 8H), 5.61 (s, 2H), 5.14 (s, 2H), 4.57 (s, 2H), 4.36 (q, 2H, *J* = 7.3), 4.10 (s, 2H), 1.38 (t, 3H, *J* = 7.3). ^{13}C NMR (50 MHz, MeOD/TFA) δ 170.0 (C), 163.0 (d, 2 × C, *J* = 250.0), 164.1 (C), 159.1, 150.4 (2 × C), 149.3 (CH), 146.0, 143.1 (2 ×C), 132.9 (s, CH), 132.0 (C), 129.7, 129.3 (7 × CH), 121.0

(C), 115.7 (2 × CH), 113.7 (C), 112.8 (d, 2 × CH, J_1 = 22.7), 111.2 (t, 1 × C, J = 18.2), 101.9 (C), 62.1, 60.0, 56.9, 50.7, 44.8 (5 ×CH_2), 41.6 (CH_3).

2-(*N*-((6-(Ethoxycarbonyl)-8-(2, 6-difluorobenzyl)-2-(4-(3-ethylureido)phenyl)-5, 8-dihydro-5-oxo imidazo[1, 2-a]pyrimidin-3-yl)methyl)-*N*-benzylamino)acetic acid (18). To a solution of **17** (2.61 g, 4.30 mmol) in pyridine (30 mL) was added ethyl isocyanate (1.4 mL, 21.5 mmol). The mixture was stirred for 18 h and subsequently water (30 mL) was added. The mixture was stirred for 1 h and the volatiles were removed. The residue was redissolved in DCM (200 mL) and the solution washed with aqueous $NaHCO_3$ (10%, 100 mL), dried (Na_2SO_4) and concentrated. Column chromatography of the residue (0% to 50% MeOH in EtOAc) afforded off-white crystals (2.05 g, 3.1 mmol, 71%). ESI-MS *m/z*: 673.5 $[M + H]^+$, 695.3 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$/TFA) δ 8.56 (s, 1H), 7.49 – 7.13 (m, 10H), 7.04 (t, 2H, J = 8.0) 5.51 (s, 2H), 5.04 (s, 2H), 4.48 (s, 2H), 4.33 (q, 2H, J = 6.8), 4.04 (s, 2H), 3.26 (q, 2H, J = 7.2), 1.36 (t, 3H, J = 7.2), 1.16 (t, 3H, J = 7.6). ^{13}C NMR (400 MHz, $CDCl_3$/TFA) δ 167.2, 162.8 (2 × C), 161.3 (dd, 2 × C, J = 251.0), 158.6, 156.3 (2 × C), 147.8 (CH), 146.0, 142.4, 140.3 (4 × C), 132.2, 129.8, 129.2, 128.9 (8 × CH), 128.2 (2 × C), 119.9 (2 × CH), 111.8 (d, 2 × CH, J = 23.0), 108.8, 101.1 (2 × C), 61.6, 59.5, 49.7, 43.7, 38.5, 34.9 (6 × CH_2), 14.6, 13.8 (2 × CH_3). HRMS *m/z* calcd for $C_{35}H_{34}N_6O_6F_2$ + H^+: 673.25807, obsd 673.25730.

8-(2, 6-Difluorobenzyl)-5, 8-dihydro-2-(4-ethylaminocarbonylaminophenyl)-3-(*N*-propargyl amidomethyl-*N*-benzylaminometyl)-5-oxoimidazo[1, 2-a]pyrimidine-6-carboxylic acid ethyl ester (4). To a solution of **18** (1.0 g, 1.5 mmol) and propargylamine (134 µL, 1.95mmol) in DMF (50 ml) were added BOP (0.88 g, 1.95 mmol) and DiPEA (0.76 mL, 4.5 mmol) and the mixture was allowed to stir for 18h. The solution was evaporated and dissolved in DCM/MeOH mixture (200 ml, 9/1 v/v) and washed with $NaHSO_3$ (10%, 100ml), $NaHCO_3$ (10%, 100ml) and water (100ml). The organic layer was dried ($MgSO_4$) and evaporated. The crude product was purified by column chromatography (50% to 100% EtOAc in toluene) to yield an off-white solid which was additionally crystallized from MeOH/$CHCl_3$ and Et_2O to provide an off-white solid (573 mg, 0.81 mmol, 54%). An analytical pure sample for biological evaluation was prepared by an additional purification on a semi-preperative RP-HPLC system (linear gradient of 5.0 CV; 30 to 45%B). ESI-MS *m/z*: 710.6 $[M + H]^+$. ^{1}H NMR (500 MHz, DMSO-*d*6/TFA) δ 9.03 (s, 1H), 8.95 (s, 1H), 8.71 (br s, 1H), 7.60 – 7.42 (m, 10H), 7.17 (t, 2H, J = 8.0), 6.65 (t, 1H), 6.25 (br s, 1H), 5.67 (s, 2H), 5.60 (s, 2H), 5.13 – 5.10 (m, 1H), 4.82 – 4.79 (m, 1H), 4.58 – 4.56 (m, 1H), 4.38 – 4.34 (m, 3H), 3.92 – 3.90 (m, 2H), 3.16 – 3.11 (m, 3H), 1.34 (t, 3H, J = 7.2), 1.08 (t, 3H, J = 7.5). ^{13}C NMR (500 MHz, DMSO/TFA) δ 163.9, 163.0 (2 × C), 161.2 (dd, 2 × C, J_1 = 250.0, J_2 = 7.5), 157.9, 155.4 (2 × C), 149.7 (CH), 144.5, 142.7, 141.8 (3 × C), 131.9 (t, 1 × CH, J = 10.0), 131.0 (2 × CH), 129.9 (C), 129.8, 129.3, 128.9 (5 × CH), 124.0 (C), 117.9 (2 × CH), 112.1 (d, 2 × CH, J = 20.0), 112.1 (C), 110.8 (t, 1 × C, J = 18.8), 101.1 (C), 79.7 (CH), 74.0 (C), 60.9, 59.0, 49.6, 44.2, 38.5, 34.2, 28.3 (7 × CH_2), 15.5, 14.3 (2 × CH_3). HRMS *m/z* calcd for $C_{38}H_{37}N_7O_5F_2$ + H^+: 710.28970, obsd 710.28799.

General procedure for di-tosylated polyethyleneglycols 20b-e.

Polyethylene glycol **19** (150 mmol) was dissolved in DCM (150 mL). *p*-Toluenesulfonyl chloride (2 eq. 300 mmol, 57.2 g) was added and the mixture was cooled to 0°C with an ice bath. Powdered KOH (8 eq. 1.2 mol, 67.2 g) was carefully added in small portions so that the mixture was kept below 5°C. After stirring for 3 hours at 0 °C, DCM (150 mL) and ice-water (300 mL) were added. The organic layer was separated and the water layer was extracted with DCM (2 × 150 mL). The combined organic layers were washed with water (1 × 100 mL), dried ($MgSO_4$) and evaporated to yield di-tosylated polyethyleneglycols quantitatively, which were used without further purification.

1, 5-Ditosyl-3-oxapentane (20b). 1H NMR (200 MHz, CDCl3) δ 7.78 (d, 4H, *J* = 8.4), 7.35 (d, 4H, *J* = 7.7), 4.11 – 4.07 (m, 4H), 3.63 – 3.58 (m, 4H), 2.45 (s, 6H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 144.9, 132.7 (4 × C), 129.8, 127.8 (8 × CH), 69.0, 68.6 (4 × CH_2), 21.5 (2 × CH_3).

1, 8-Ditosyl-3, 6-dioxaoctane (20c). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.78 (d, 4H, *J* = 8.0), 7.34 (d, 4H, *J* = 8.0), 4.16 – 4.11 (m, 4H), 3.67 – 3.62 (m, 4H), 3.52 (s, 4H), 2.44 (s, 6H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 144.7, 132.7 (4 × C), 129.8, 127.8 (8 × CH), 70.5, 69.1, 68.4 (6 × CH_2), 21.5 (2 × CH_3).

1, 11-Ditosyl-3, 6, 9 -trioxaundecane (20d). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.78 (d, 4H, *J* = 8.0), 7.34 (d, 4H, *J* = 8.4), 4.17 – 4.13 (m, 8H), 3.67 – 3.62 (m, 4H), 3.55 (s, 4H), 2.44 (s, 6H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 144.7, 132.7 (4 × C), 129.7, 127.8 (8 × CH), 70.5, 70.3, 69.1, 68.4 (8 × CH_2), 21.5 (2 × CH_3).

1, 14-Ditosyl-3, 6, 9, 12-tetraoxatetradecane (20e). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.78 (d, 4H, *J* = 8.0), 7.34 (d, 4H, *J* = 8.4), 4.17 – 4.13 (m, 12H), 3.67 – 3.62 (m, 4H), 3.55 (s, 4H), 2.44 (s, 6H).^{13}C NMR (50 MHz, $CDCl_3$) δ 144.7, 132.7 (4 × C), 129.7, 127.8 (8 × CH), 70.6 70.5, 70.3, 69.1, 68.4 (10 × CH_2), 21.5 (2 × CH_3).

1, 2-Diazidoethane (21a). ***Caution: Because of the explosive nature of low molecular weight diazides, 1, 2-diazidoethane was not isolated, but stored as a solution in DMF.*** 1, 2-dichloroethane (150 mmol, 15.2 g) was dissolved in DMF (225 mL). NaN_3 (600 mmol, 39.0 g) and TBAI (5 mol%, 7.5 mmol, 2.8 g) were added and the mixture was heated at 80°C for 18 hours. Et_2O was added and the resulting solids were filtered. The Et_2O was evaporated and the concentration of the final solution of 1, 2-diazidoethane in DMF was determined by ^{1}H NMR and was used without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.53 (s, 4H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 49.0 (2 × CH_2). ATR-IR (thin film): 2100 (N_3) cm^{-1}.

General procedure for the synthesis of bis-azide ethyleneglycols 21.
Ditosylated ethyleneglycol **20** (150 mmol) was dissolved in DMF (225 mL). NaN_3 (600 mmol, 39.0 g) and TBAI (5 mol%, 7.5 mmol, 2.8 g) were added and the mixture was heated at 80°C for 18 hours. The DMF was evaporated and the solid residue was suspended in Et_2O. The insoluble salts were filtered and the filtrate concentrated. This procedure was repeated twice until all the salts and DMF were removed. A slightly yellow liquid remained in quantitative yield.

1, 5-Diazido-3, 6-oxapentane (21b). ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.67 (t, 4H, *J* = 4.7), 3.40 (t, 4H, *J* = 5.1). ^{13}C NMR (50 MHz, $CDCl_3$) δ 69.4, 50.4 (4 × CH_2). ATR-IR (thin film): 2089 (N_3), 1124 (C-O) cm^{-1}.

1, 8-Diazido-3, 6-dioxaoctane (21c). ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.64 – 3.56 (m, 8H), 3.31 (t, 4H, *J* = 5.1). ^{13}C NMR (50 MHz, $CDCl_3$) δ 70.4, 69.8, 50.4 (6 × CH_2). ATR-IR (thin film): 2095 (N_3), 1115 (C-O) cm^{-1}.

1, 11-Diazido-3, 6, 9-trioxaundecane (21d). ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.56 – 3.54 (m, 12H), 3.27 (t, 4H, *J* = 5.1). ^{13}C NMR (50 MHz, $CDCl_3$) δ 70.3, 69.6, 50.3 (8 × CH_2). ATR-IR (thin film): 2091 (N_3), 1090 (C-O) cm^{-1}.

1, 14-Diazido-3, 6, 9, 12-tetraoxatetradecane (21e). ^{1}H NMR (200 MHz, $CDCl_3$) δ 3.62 – 3.60 (m, 16H), 3.34 (t, 4H, *J* = 5.1). ^{13}C NMR (50 MHz, $CDCl_3$) δ 70.5, 69.9, 50.5 (10 × CH_2). ATR-IR (thin film): 2095 (N_3), 1113 (C-O) cm^{-1}.

General method for the preparation of monomeric ligands 22a-e, 23a-e and 24a-e.
To a solution of the desired acetylene functionalized ligand **2**, **3** or **4** (0.05 mmol) and bis-azide spacer **21a, 21b, 21c, 21d or 21e** (0.25 mmol) in a mixture of degassed *t*BuOH/CH_3CN/H_2O (2/1/1; v/v/v, 900 µL) were added sodium ascorbate (1 eq. 50 µL of a 1M solution in H_2O) and $CuSO_4$ (0.2 eq. 50 µL of a 0.2M solution in H_2O). The reaction mixture was stirred and heated at 60°C until RP-HPLC showed complete conversion of the starting material. The mixture was evaporated and redissolved in dioxane/H_2O/CH_3CN/TFA (10/1/1/1; v/v/v/v, 2 mL). The crude products were analyzed by LC-MS and purified by semi-preparative RP-HPLC (linear gradient of 5.0 CV; 30 to 45% B). Evaporation and lyophilization of the combined fractions furnished monomeric ligands **22a-e, 23a-e and 24a-e** as white amorphous powders.

Monomeric ligand 22a. Yield after RP-HPLC purification: 12.6 mg (14.7 µmol, 30%). LC-MS analysis: t_R 6.94 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 750.4 $[M + H]^+$). ^1H NMR (500 MHz, DMSO-*d*6) δ 9.14 (t, 1H, *J* = 5.0), 9.04 (s, 1H), 8.73 (s, 1H), 8.59 (br s, 1H), 8.13 (s, 1H), 7.54 (dd, 4H, *J* = 24.5, *J* = 9.0), 7.49 – 7.42 (m, 6H), 7.17 (t, 2H, *J* = 8.5), 6.28 (br s, 1H), 5.69 (s, 2H), 5.15 – 5.11 (m, 1H), 4.81 – 4.76 (m, 1H,), 4.71, 4.69 (2 × d, 1H, *J* = 5.5), 4.62 – 4.50 (m, 4H), 4.40 – 4.36 (m, 1H), 3.84 (t, 2H, *J* = 5.4), 3.11 (q, 2H, *J* = 5.2), 2.46 (s, 3H), 1.06 (t, 3H, *J* = 7.2). HRMS *m/z* calcd for $C_{37}H_{37}N_{13}O_3F_2 + H^+$: 750.31654, obsd 750.31831.

Monomeric ligand 22b. Yield after RP-HPLC purification: 16.1 mg (17.7 µmol, 35%). LC-MS analysis: t_R 6.82 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 794.5 $[M + H]^+$). ^1H NMR (500 MHz, DMSO-*d*6) δ 9.12 (t, 1H, *J* = 5.0), 9.01 (s, 1H), 8.78 (s, 1H), 8.61 (br s, 1H), 8.04 (s, 1H), 7.55 (dd, 4H, J_1 = 14.5, J_2 = 8.7), 7.50 – 7.42 (m, 6H), 7.16 (t, 2H, *J* = 8.1), 6.33 (br s, 1H), 5.70 (s, 2H), 5.15 – 5.11 (m, 1H), 4.81 – 4.76 (m, 1H), 4.63 (ddd, 2H, J_1 = 32.9, J_2 = 15.2, J_3 = 5.7), 4.57 (t, 2H, *J* = 5.1), 4.54 – 4.53 (m, 1H), 4.46 – 4.40 (m, 1H), 3.86 (t, 2H, *J* = 5.2), 3.59 (t, 2H, *J* = 4.8), 3.36 (t, 2H, *J* = 5.0), 3.11 (q, 2H, *J* = 5.2), 2.46 (s, 3H), 1.05 (t, 3H, *J* = 7.2). HRMS *m/z* calcd for $C_{39}H_{41}N_{13}O_4F_2 + H^+$: 794.34453, obsd 794.34299.

Monomeric ligand 22c. Yield after RP-HPLC purification: 22.3 mg (23.4 µmol, 47%). LC-MS analysis: t_R 7.25 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 838.4 $[M + H]^+$). ^1H NMR (500 MHz, DMSO-*d*6) δ 9.13 (t, 1H, *J* = 5.2), 9.01 (s, 1H), 8.84 (s, 1H), 8.63 (br s, 1H), 8.05 (s, 1H), 7.55 (dd, 4H, J_1 = 20.9, J_2 = 8.7), 7.50 – 7.41 (m, 6H), 7.16 (t, 2H, *J* = 8.2), 6.33 (br s, 1H), 5.70 (s, 2H), 5.15 – 5.11 (m, 1H), 4.82 – 4.76 (m, 1H), 4.71 – 4.63 (m, 1H), 4.61 – 4.47 (m, 2H), 4.55 (t, 2H, *J* = 5.1), 4.40 – 4.37 (m, 1H), 3.83 (t, 2H, *J* = 5.2,), 3.55 – 3.52 (m, 6H), 3.35 (t, 2H, *J* = 5.0), 3.11 (q, 2H, *J* = 7.1), 2.46 (s, 3H), 1.05 (t, 3H, *J* = 7.2). HRMS *m/z* calcd for $C_{41}H_{45}N_{13}O_5F_2 + H^+$: 838.37074, obsd 838.36890.

Monomeric ligand 22d. Yield after RP-HPLC purification: 15.8 mg (15.8 µmol, 32%). LC-MS analysis: t_R 5.75 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 882.4 $[M + H]^+$). ^1H NMR (500 MHz, DMSO-*d*6) δ 9.13 (t, 1H, *J* = 5.2), 9.02 (s, 1H), 8.83 (s, 1H), 8.62 (br s, 1H), 8.05 (s, 1H), 7.55 (dd, 4H, J_1 = 21.8, J_2 = 8.7), 7.50 – 7.41 (m, 6H), 7.16 (t, 2H, *J* = 8.2), 6.33 (br s, 1H,), 5.70 (s, 2H), 5.15 – 5.11 (m, 1H), 4.82 – 4.76 (m, 1H), 4.69 (dd, 2H, J_1= 15.1, J_2 = 5.8), 4.62 – 4.52 (m, 2H), 4.55 (t, 2H, *J* = 5.2), 4.40 – 4.37 (m, 1H), 3.83 (t, 2H, *J* = 5.2), 3.55 – 3.52 (m, 10H), 3.36 (t, 2H, *J* = 5.0), 3.11 (q, 2H, *J* = 6.9), 2.46 (s, 3H), 1.06 (t, 3H, *J* = 7.2). HRMS *m/z* calcd for $C_{43}H_{49}N_{13}O_6F_2 + H^+$: 882.39696, obsd 882.39498.

Monomeric ligand 22e. Yield after RP-HPLC purification: 18.6 mg (17.9 µmol, 36%). LC-MS analysis: t_R 7.24 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 926.2 $[M + H]^+$). ^1H NMR (500 MHz, DMSO-*d*6) δ 9.13 (t, 1H, *J* = 5.2), 9.02 (s, 1H), 8.83 (s, 1H), 8.61 (br s, 1H), 8.07 (s, 1H), 7.55 (dd, 4H, J_1 = 17.8, J_2 = 8.7), 7.50 – 7.41 (m, 6H), 7.16 (t, 2H, *J* = 8.2), 6.33 (br s, 1H), 5.70 (s, 2H), 5.15 – 5.11 (m, 1H), 4.82 – 4.76 (m, 1H), 4.69 (dd, 2H, J_1 =

15.1, J_2 = 5.8), 4.62 – 4.52 (m, 2H), 4.55 (t, 2H, *J* = 5.2), 4.40 – 4.37 (m, 1H,), 3.82 (t, 2H, *J* = 5.2), 3.55 – 3.52 (m, 14H), 3.36 (t, 2H, *J* = 5.0), 3.11 (q, 2H, *J* = 7.1), 2.46 (s, 3H), 1.05 (t, 3H, *J* = 7.2). HRMS *m/z* calcd for $C_{45}H_{53}N_{13}O_7F_2$ + H^+: 926.42317, obsd 926.41971.

Monomeric ligand 23a. Yield after RP-HPLC purification: 6.9 mg (8.0 µmol, 16%). LC-MS analysis: t_R 7.21 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 751.4 $[M + H]^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 9.01 (s, 1H), 8.92 (s, 1H), 8.72 (br s, 1H) 8.01 (s, 1H), 7.53 (dd, 4H, *J* = 11.2, *J* = 9.5), 7.48 – 7.35 (m, 6H), 7.17 (t, 2H, *J* = 8.1), 6.81 (m, 1H), 5.65 (s, 2H), 5.11 – 5.08 (m, 1H), 4.80 – 4.76 (m, 1H), 4.56 – 4.51 (m, 1H), 4.54 (t, 2H, *J* = 5.2), 4.38 – 4.27 (m, 5H), 3.82 (t, 2H, *J* = 5.2), 2.48 (s, 3H), 1.13 (t, 3H, *J* = 7.1). HRMS *m/z* calcd for $C_{37}H_{36}N_{12}O_4F_2$ + H^+: 751.30233, obsd 751.30002.

Monomeric ligand 23b. Yield after RP-HPLC purification: 7.2 mg (7.9 µmol, 16%). LC-MS analysis: t_R 7.34 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 795.3 $[M + H]^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 9.01 (s, 1H), 8.89 (s, 1H), 8.71 (br s, 1H) 7.96 (s, 1H), 7.55 – 7.40 (dd, 4H, *J* = 12.6, *J* = 9.1), 7.48 – 7.40 (m, 6H), 7.17 (t, 2H, *J* = 8.1), 6.78 (t, 1H, *J* = 5.5), 5.65 (s, 2H), 5.11 – 5.08 (m, 1H), 4.80 – 4.76 (m, 1H), 4.56 – 4.51 (m, 1H), 4.52 (t, 2H, *J* = 5.2), 4.38 – 4.27 (m, 5H), 3.83 (t, 2H, *J* = 5.2), 3.58 (t, 2H, *J* = 5.0), 3.36 (t, 2H, *J* = 4.8), 2.48 (s, 3H), 1.13 (t, 3H, *J* = 7.1). HRMS *m/z* calcd for $C_{39}H_{40}N_{12}O_5F_2$ + H^+: 795.32855, obsd 795.32597.

Monomeric ligand 23c. Yield after RP-HPLC purification: 14.7 mg (15.4 µmol, 31%). LC-MS analysis: t_R 7.38 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 839.3 $[M + H]^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 9.01 (s, 1H), 8.89 (s, 1H), 8.71 (br s, 1H) 7.91 (s, 1H), 7.53 – 7.35 (m, 10H), 7.16 (t, 2H, *J* = 8.2), 6.75 (br s, 1H), 5.64 (s, 2H), 5.11 – 5.08 (m, 1H), 4.80 – 4.76 (m, 1H), 4.56 – 4.44 (m, 3H), 4.39 – 4.27 (m, 5H), 3.75 (t, 2H, *J* = 5.0), 3.58 – 3.47 (m, 10H), 3.38 (t, 2H, *J* = 5.1), 2.48 (s, 3H), 1.33 (t, 3H, *J* = 7.1). HRMS *m/z* calcd for $C_{41}H_{44}N_{12}O_6F_2$ + H^+: 839.35476, obsd 839.35210.

Monomeric ligand 23d. Yield after RP-HPLC purification: 14.7 mg (12.9 µmol, 26%). LC-MS analysis: t_R 5.90 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 883.3 $[M + H]^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 9.01 (s, 1H), 8.89 (s, 1H), 8.71 (br s, 1H) 7.93 (s, 1H), 7.53 (dd, 4H, *J* = 13.0, *J* = 9.0), 7.48 – 7.40 (m, 6H), 7.17 (t, 2H, *J* = 8.2), 6.76 (br s, 1H), 5.65 (s, 2H), 5.11 – 5.08 (m, 1H), 4.80 – 4.76 (m, 1H), 4.56 – 4.54 (m, 1H), 4.50 (t, 2H, *J* = 5.2), 4.39 – 4.27 (m, 5H), 3.80 (t, 2H, *J* = 5.0), 3.58 – 3.47 (m, 14H, *J* = 5.0), 3.35 (t, 2H, *J* = 4.8), 2.48 (s, 3H), 1.33 (t, 3H, *J* = 7.1). RMS *m/z* calcd for $C_{43}H_{48}N_{12}O_7F_2$ + H^+: 883.38097, obsd 883.37797.

Monomeric ligand 23e. Yield after RP-HPLC purification: 10.4 mg (10.0 µmol, 20%). LC-MS analysis: t_R 7.38 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 927.5 $[M + H]^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 9.01 (s, 1H), 8.88 (s, 1H), 8.71 (br s, 1H) 7.93 (s, 1H), 7.53 (dd, 4H, *J* = 12.4, *J* = 9.2), 7.51 – 7.40 (m, 6H), 7.17 (t, 2H, *J* = 8.2), 6.78 (br s, 1H), 5.65 (s, 2H), 5.11 – 5.08 (m, 1H), 4.80 – 4.76 (m, 1H), 4.56 – 4.54 (m, 1H), 4.50 (t, 2H, *J* = 5.2), 4.38 – 4.27 (m, 5H), 3.80 (t, 2H, *J* = 5.0), 3.58 – 3.47 (m, 10H, *J* = 5.0), 3.35 (t, 2H, *J* = 4.8), 2.48 (s, 3H), 1.33 (t, 3H, *J* = 7.1). HRMS *m/z* calcd for $C_{45}H_{52}N_{12}O_8F_2$ + H^+: 927.40719, obsd 927.40473.

Monomeric ligand 24a. Yield after RP-HPLC purification: 13.3 mg (14.2 µmol, 28.4%). LC-MS analysis: T_R 7.47 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 822.4 $[M + H]^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 8.96 (s, 1H), 8.79 (br s, 2H), 7.90 (s, 1H), 7.51 (dd, 4H, J_1 = 13.8, J_2 = 8.7), 7.49 – 7.35 (m, 3H), 7.25 – 7.20 (m, 3H), 7.15 (t, 2H, *J* = 8.2), 6.35 (br s, 1H), 5.61 (s, 2H), 5.05 – 4.76 (br s, 2H), 4.57 – 4.52 (m, 2H), 4.51 (t, 2H, *J* = 5.6), 4.31 (q, 2H, *J* = 7.1), 4.06 (br s, 2H), 3.88 – 3.80 (m, 2H), 3.78 (t, 2H, *J* = 5.7), 3.16 – 3.11 (m, 2H), 1.34 (t, 3H, *J* = 7.2), 1.05 (t, 3H, *J* = 7.2). HRMS *m/z* calcd for $C_{40}H_{41}N_{13}O_5F_2$ + H^+: 822.33944, obsd 822.33927.

Monomeric ligand 24b. Yield after RP-HPLC purification: 24.0 mg (24.5 µmol, 49%). LC-MS analysis: T_R 7.59 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 866.4 $[M + H]^+$). ^1H NMR (500 MHz, DMSO-*d*6) δ 8.96 (s, 1H), 8.83 (br s, 2H), 7.79 (s, 1H), 7.51 (dd, 4H, J_1 = 20.4, J_2 = 8.7), 7.48 – 7.32 (m, 3H), 7.32 – 7.20 (m, 3H), 7.14 (t, 2H, *J* = 8.2), 6.39 (br s, 1H), 5.61 (s, 2H), 5.05 – 4.76 (br s, 2H), 4.57 – 4.42 (m, 2H), 4.49 (t, 2H, *J* = 5.2), 4.31 (q, 2H, *J* = 7.1), 4.06 – 4.01 (br s, 2H), 3.88 – 3.82 (m, 2H), 3.80 (t, 2H, *J* = 5.7), 3.59 – 3.52 (m, 2H), 3.32 (t, 2H, *J* = 5.1) 3.16 – 3.08 (m, 2H), 1.33 (t, 3H, *J* = 7.2), 1.06 (t, 3H, *J* = 7.2). HRMS *m/z* calcd for $C_{42}H_{45}N_{13}O_6F_2 + H^+$: 866.36566, obsd 866.36566.

Monomeric ligand 24c. Yield after RP-HPLC purification: 23.1 mg (22.5 µmol, 45%). LC-MS analysis: T_R 7.71 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 910.6 $[M + H]^+$). ^1H NMR (500 MHz, DMSO-*d*6) δ 8.96 (s, 1H), 8.86 (br s, 2H), 7.81 (s, 1H), 7.51 (dd, 4H, J_1 = 18.9, J_2 = 8.7), 7.48 – 7.32 (m, 3H), 7.32 – 7.20 (m, 3H), 7.16 (t, 2H, *J* = 8.3), 6.38 (br s, 1H), 5.61 (s, 2H), 5.08 – 4.76 (br s, 2H), 4.57 – 4.42 (m, 2H), 4.47 (t, 2H, *J* = 5.2), 4.31 (q, 2H, *J* = 7.1), 4.06 – 4.01 (br s, 2H), 3.92 – 3.80 (m, 2H), 3.77 (t, 2H, *J* = 5.2), 3.55 – 3.49 (m, 6H), 3.33 (t, 2H, *J* = 5.0) 3.13 – 3.10 (m, 2H), 1.33 (t, 3H, *J* = 7.1), 1.06 (t, 3H, *J* = 7.2). HRMS *m/z* calcd for $C_{44}H_{49}N_{13}O_7F_2 + H^+$: 910.39187, obsd 910.39296.

Monomeric ligand 24d. Yield after RP-HPLC purification: 10.6 mg (9.9 µmol, 20%). LC-MS analysis: T_R 7.73 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 954.7 $[M + H]^+$). ^1H NMR (500 MHz, DMSO-*d*6) δ 8.96 (s, 1H), 8.75 (br s, 2H), 7.81 (s, 1H), 7.52 – 7.48 (m, 4H), 7.47 – 7.32 (m, 3H), 7.32 – 7.20 (m, 3H), 7.15 (t, 2H, *J* = 8.2), 6.31 (br s, 1H), 5.60 (s, 2H), 5.08 – 4.76 (br s, 2H), 4.57 – 4.42 (m, 2H), 4.46 (t, 2H, *J* = 5.2), 4.30 (q, 2H, *J* = 7.0), 4.06 – 4.01 (br s, 2H), 3.92 – 3.80 (m, 2H), 3.77 (t, 2H, *J* = 5.3), 3.58 – 3.49 (m, 10H), 3.35 (t, 2H, *J* = 5.1), 3.13 – 3.10 (m, 2H), 1.33 (t, 3H, *J* = 7.3), 1.06 (t, 3H, *J* = 7.2).

Monomeric ligand 24e. Yield after RP-HPLC purification: 22.9 mg (20.6 µmol, 41%). LC-MS analysis: T_R 7.73 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 998.5 $[M + H]^+$). ^1H NMR (500 MHz, DMSO-*d*6) δ 8.90 – 8.50 (m, 3H), 7.74 (s, 1H), 7.52 – 7.30 (m, 6H) 7.32 – 7.15 (m, 6H), 6.23 (br s, 1H), 5.53 (s, 2H), 5.08 – 4.76 (br s, 2H), 4.57 – 4.32 (m, 4H), 4.23 (q, 2H, *J* = 7.0), 4.06 – 4.95 (br s, 2H), 3.90 – 3.70 (m, 2H), 3.77 – 3.24 (m, 18H) 3.09 – 3.03 (m, 2H), 1.26 (t, 3H, *J* = 7.3), 1.00 (t, 3H, *J* = 7.2). HRMS *m/z* calcd for $C_{48}H_{57}N_{13}O_9F_2 + H^+$: 998.44430, obsd 998.44574.

General method for the preparation of dimeric ligands 25a-e, 26a-e and 27a-e.

To a solution of the desired acetylene functionalized ligand **2**, **3** or **4** (0.10 mmol) and bis-azide spacer **21a, 21b, 21c, 21d or 21e** (0.05 mmol) in a mixture of degassed tBuOH/MeCN/H_2O (2/1/1; v/v/v, 800 µL) were added sodium ascorbate (1 eq. 100 µL of a 1M solution in H_2O) and $CuSO_4$ (0.2 eq. 100 µL of a 0.2M solution in H_2O). The reaction mixture was stirred and heated at 60°C until RP-HPLC showed complete conversion of the starting material. The mixture was evaporated and redissolved in Dioxane/H_2O/CH_3CN/TFA (10/1/1/1; v/v/v/v, 2 mL). The crude products were analyzed by LC-MS and purified by semi-preparative RP-HPLC (linear gradient of 5.0 CV; 30 to 45% B). Evaporation and lyophilization of the combined fractions furnished dimeric ligands **25a-e, 26a-e and 27a-e** as white amorphous powders.

Dimeric ligand 25a. Yield after RP-HPLC purification: 25.7 mg (15.9 µmol, 32%). LC-MS analysis: t_R 6.87 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1387.6 $[M + H]^+$). ^1H NMR (500 MHz, DMSO-*d*6) δ 9.11 (t, 2H, *J* = 5.3), 8.99 (s, 2H), 8.71 (s, 2H), 8.59 (br s, 2H), 8.02 (s, 2H), 7.54 (dd, 8H, J_1 = 23.7, J_2 = 8.7), 7.49 – 7.40 (m, 12H), 7.16 (t, 4H, *J* = 8.2), 6.25 (t, 2H, *J* = 5.2), 5.69 (s, 4H), 5.13 – 5.10 (m, 2H), 4.92 (s, 4H), 4.79 – 4.70 (m, 2H), 4.68

– 4.59 (m, 2H), 4.57 – 4.50 (m, 4H), 4.40 – 4.36 (m, 2H), 3.14 – 3.09 (m, 4H), 2.46 (s, 6H), 1.05 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{72}H_{70}N_{20}O_6F_4$ + H^+: 1387.57961, obsd 1387.59127.

Dimeric ligand 25b. Yield after RP-HPLC purification: 29.8 mg (18.0 µmol, 36%). LC-MS analysis: t_R 7.02 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1431.8 [M + H]$^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 9.12 (s, 2H), 9.02 (s, 2H), 8.78 (s, 2H), 8.62 (br s, 2H), 7.95 (s, 2H), 7.53 (dd, 8H, J_1 = 19.5, J_2 = 6.7), 7.49 – 7.40 (m, 12H), 7.15 (t, 4H, *J* = 8.2), 6.34 (br s, 2H), 5.69 (s, 4H), 5.13 – 5.10 (m, 2H), 4.79 – 4.70 (m, 2H), 4.68 – 4.64 (m, 2H), 4.57 – 4.46 (m, 8H), 4.40 – 4.36 (m, 2H), 3.83 – 3.77 (m, 4H), 3.11 (q, 4H, *J* = 7.2), 2.46 (s, 6H), 1.05 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{74}H_{74}N_{20}O_7F_4$ + H^+: 1431.60582, obsd 1431.61762.

Dimeric ligand 25c. Yield after RP-HPLC purification: 11.1 mg (6.5 µmol, 13%). LC-MS analysis: T_R 7.20 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1475.8 [M + H]$^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 9.14 (t, 2H, *J* = 5.3), 9.02 (s, 2H), 8.76 (s, 2H), 8.67 – 8.58 (m, 2H), 8.05 (s, 2H), 7.59 – 7.42 (m, 20H), 7.17 (t, 4H, *J* = 8.2), 6.38 – 6.42 (m, 2H), 5.70 (s, 4H), 5.13 (d, 2H, *J* = 13.5), 4.82 – 4.78 (m, 2H), 4.71 – 4.58 (ddd, 4H, J_1 = 5.1, J_2 = 15.1, J_3 = 42.5), 4.54 – 4.46 (m, 6H), 4.49 – 4.37 (m, 2H), 3.80 (t, 4H, *J* = 5.1), 3.52 (s, 4H), 3.14 – 3.10 (m, 4H), 2.48 – 2.46 (m, 6H), 1.07 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{76}H_{78}N_{20}O_8F_4$ + H^+: 1475.63204, obsd 1475.64714.

Dimeric ligand 25d. Yield after RP-HPLC purification: 14.1 mg (8.1 µmol, 16%). LC-MS analysis: T_R 7.25 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1519.7 [M + H]$^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 9.12 (br s, 2H), 9.00 (s, 2H), 8.82 (s, 2H), 8.67 – 8.58 (br s, 2H), 8.05 (s, 2H), 7.55 (dd, 8H, J_1 = 20.0, J_2 = 8.8), 7.50 – 7.38 (m, 12H), 7.17 (t, 4H, *J* = 8.2), 6.37 (br s, 2H), 5.69 (s, 4H), 5.13 – 5.11 (m, 2H), 4.81 – 4.78 (m, 2H), 4.71 – 4.61 (m, 2H), 4.59 – 4.42 (m, 8H), 4.40 – 4.34 (m, 2H), 3.81 (t, 4H, *J* = 5.4), 3.52 – 3.47 (m, 4H), 3.56 – 3.49 (m, 4H), 3.16 – 3.09 (m, 4H), 2.48 (br s, 6H), 1.05 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{78}H_{82}N_{20}O_9F_4$ + H^+: 1519.65825, obsd 1519.66753.

Dimeric ligand 25e. Yield after RP-HPLC purification: 13.8 mg (7.7 µmol, 15%). LC-MS analysis: T_R 7.27 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1563.9 [M + H]$^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 9.12 (br s, 2H), 9.01 (s, 2H), 8.79 (s, 2H), 8.65 – 8.57 (br s, 2H), 8.05 (s, 2H), 7.55 (dd, 8H, J_1= 21.9, J_2 = 8.7), 7.50 – 7.38 (m, 12H), 7.16 (t, 4H, *J* = 8.2), 6.34 (br s, 2H), 5.69 (s, 4H), 5.13 – 5.08 (m, 2H), 4.81 – 4.75 (m, 2H), 4.71 – 4.64 (m, 2H), 4.59 – 4.42 (m, 8H), 4.40 – 4.36 (m, 2H), 3.81 (t, 4H, *J* = 5.5), 3.57 – 3.43 (m, 12H), 3.14 – 3.09 (m, 4H), 2.46 (br s, 6H), 1.05 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{80}H_{86}N_{20}O_{10}F_4$ + H^+: 1563.68447, obsd 1563.68651.

Dimeric ligand 26a. Yield after RP-HPLC purification: 27.9 mg (17.3 µmol, 35%). LC-MS analysis: t_R7.50 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1389.5 [M + H]$^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 9.01 (s, 2H), 8.98 (s, 2H), 8.73 (br s, 2H), 7.90 (s, 2H), 7.55 – 7.51 (m, 8H), 7.48 – 7.40 (m, 12H), 7.16 (t, 4H, *J* = 8.3), 6.85 (m, 2H), 5.65 (s, 4H), 5.11 – 5.08 (m, 2H), 4.87 (s, 4H) 4.81 – 4.77 (m, 2H), 4.56 – 4.51 (m, 2H), 4.37 – 4.29 (m, 10H), 2.48 (s, 6H), 1.13 (t, 6H, *J* = 7.1). HRMS *m/z* calcd for $C_{72}H_{68}N_{18}O_8F_4$ + H^+: 1389.54764, obsd 1389.55646.

Dimeric ligand 26b. Yield after RP-HPLC purification: 29.7 mg (17.9 µmol, 36%). LC-MS analysis: t_R 7.62 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1433.7 [M + H]$^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 9.00 (s, 2H), 8.98 (s, 2H), 8.70 (br s, 2H), 7.86 (s, 2H), 7.52 (dd, 8H, *J* = 11.4, *J* = 9.4), 7.48 – 7.34 (m, 12H), 7.16 (t, 4H, *J* = 8.3), 6.73 (t, 2H, *J* = 5.0), 5.64 (s, 4H,), 5.10 – 5.08 (m, 2H), 4.79 – 4.75 (m, 2H), 4.56 – 4.54 (m, 2H), 4.37 – 4.29 (m, 10H), 3.79 (t, 4H, *J* = 5.2), 2.48 (s, 6H), 1.33 (t, 6H, *J* = 7.3). HRMS *m/z* calcd for $C_{74}H_{72}N_{18}O_9F_4$ + H^+: 1433.57385, obsd 1433.58036.

Dimeric ligand 26c. Yield after RP-HPLC purification: 25.6 mg (15.0 µmol, 30%). LC-MS analysis: t_R 7.64 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1477.7 $[M + H]^+$). ^1H NMR (400 MHz, DMSO-*d*6) δ 8.99 (s, 2H), 8.82 (s, 2H), 8.69 (br s, 2H), 7.90 (s, 2H), 7.80 (s, 2H), 7.55 – 7.32 (m, 12H,), 7.25 – 7.10 (m, 10H), 6.70 (br s, 2H), 5.62 (s, 4H), 5.10 – 5.05 (m, 2H), 4.79 – 4.70 (m, 2H, 4.56 – 4.45 (m, 6H), 4.37 – 4.10 (m, 10H), 3.73 (t, 4H, *J* = 4.9), 3.43 (s, 4H), 2.48 (s, 6H), 1.30 (s, 6H). HRMS *m/z* calcd for $C_{76}H_{76}N_{18}O_{10}F_4 + H^+$: 1477.60007, obsd 1477.61412.

Dimeric ligand 26d. Yield after RP-HPLC purification: 26.0 mg (14.9 µmol, 30%). LC-MS analysis: t_R 5.89 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1522.5 $[M + H]^+$). ^1H NMR (400 MHz, DMSO-*d*6) δ 9.00 (s, 2H), 8.83 (s, 2H), 8.68 (br s, 2H), 7.91 (s, 2H), 7.82 – 7.78 (m, 2H), 7.55 – 7.35 (m, 12H), 7.25 – 7.10 (m, 10H), 6.70 (br s, 2H), 5.59 (s, 4H), 5.10 – 5.05 (m, 2H), 4.80 – 4.70 (m, 2H), 4.56 – 4.40 (m, 6H), 4.37 – 4.15 (m, 10H), 3.76 (t, 4H, *J* = 5.0), 3.55 – 3.45 (m, 8H), 2.48 (s, 6H), 1.30 (s, 6H). HRMS *m/z* calcd for $C_{78}H_{80}N_{18}O_{11}F_4 + H^+$: 1521.62628, obsd 1521.64257.

Dimeric ligand 26e. Yield after RP-HPLC purification: 31.1 mg (17.3 µmol, 35%). LC-MS analysis: t_R 5.91 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1565.6 $[M + H]^+$). ^1H NMR (400 MHz, DMSO-*d*6) δ 9.00 (s, 2H), 8.83 (s, 2H), 8.69 (br s, 2H), 7.92 (s, 2H), 7.82 – 7.78 (m, 2H), 7.58 – 7.35 (m, 12H), 7.25 – 7.10 (m, 10H), 6.68 (br s, 2H), 5.59 (s, 4H), 5.10 – 5.05 (m, 2H), 4.80 – 4.70 (m, 2H), 4.56 – 4.40 (m, 6H), 4.37 – 4.15 (m, 10H), 3.77 (t, 4H, *J* = 5.1), 3.55 – 3.45 (m, 12H), 2.48 (s, 6H), 1.30 (s, 6H). HRMS *m/z* calcd for $C_{80}H_{84}N_{18}O_{12}F_4 + H^+$: 1565.65250, obsd 1565.66724.

Dimeric ligand 27a. Yield after RP-HPLC purification: 15.3 mg (8.7 µmol, 17%). LC-MS analysis: T_R 7.94 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1531.7 $[M + H]^+$). ^1H NMR (600 MHz, DMSO-*d*6) δ 8.97 (s, 2H), 8.82(br s, 4H), 7.82 (s, 2H), 7.50 (dd, 8H, J_1 = 25.4, J_2 = 8.5,), 7.45 – 7.41 (m, 4H), 7.39 – 7.35 (m, 4H), 7.28 – 7.20 (m, 6H), 7.14 (t, 2H, *J* = 8.1), 6.38 (br s, 2H), 5.60 (s, 4H), 4.89 (br s, 4H), 4.80 (s, 4H), 4.50 (br s, 4H), 4.29 (q, 4H, *J* = 6.9,), 4.05 (br s, 4H), 3.88 (br s, 4H), 3.11 – 3.09 (m, 4H), 1.32 (t, 6H, *J* = 7.1), 1.05 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{78}H_{78}N_{20}O_{10}F_4 + H^+$: 1531.62187, obsd 1531.62231.

Dimeric ligand 27b. Yield after RP-HPLC purification: 41.9 mg (23.2 µmol, 46%). LC-MS analysis: T_R 8.04 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1575.5 $[M + H]^+$). ^1H NMR (600 MHz, DMSO-*d*6) δ 8.91 (s, 2H), 8.67 (br s, 4H), 7.71 (s, 2H), 7.47 – 7.11 (m, 20H), 6.21 (br s, 2H), 5.56 (s, 4H, 4.89 (br s, 4H), 4.50 (br s, 4H), 4.39 (t, 4H, *J* = 5.2), 4.25 (q, 4H, *J* = 6.9), 4.05 (br s, 4H,), 3.88 (br s, 4H), 3.70 (m, 4H), 3.09 – 3.07 (m, 4H), 1.31 (t, 6H, *J* = 7.1), 1.05 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{80}H_{82}N_{20}O_{11}F_4 + H^+$: 1575.64808, obsd 1575.67161.

Dimeric ligand 27c. Yield after RP-HPLC purification: 13.0 mg (7.0 µmol, 14.0%). LC-MS analysis: T_R 7.86 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1619.8 $[M + H]^+$). ^1H NMR (500 MHz, DMSO-*d*6) δ 8.96 (s, 2H), 8.81(br s, 4H), 7.79 (s, 2H), 7.50 (dd, 8H, J_1 = 21.1, J_2 = 8.7), 7.47 – 7.41 (m, 4H), 7.39 – 7.35 (m, 4H), 7.28 – 7.19 (m, 6H), 7.15 (t, 2H, *J* = 8.2), 6.36 (br s, 2H,), 5.60 (s, 4H), 4.89 (br s, 4H), 4.50 (br s, 4H), 4.42 (t, 4H, *J* = 5.2), 4.29 (q, 4H, *J* = 6.9), 4.05 (br s, 4H), 3.88 (br s, 4H), 3.70 (t, 4H, *J* = 5.2), 3.43 (s, 4H), 3.13 – 3.09 (m, 4H), 1.31 (t, 6H, *J* = 7.1), 1.05 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{82}H_{86}N_{20}O_{12}F_4 + H^+$: 1619.67430, obsd 1619.68708.

Dimeric ligand 27d. Yield after RP-HPLC purification: 32.1 mg (17.0 µmol, 34.0%). LC-MS analysis: T_R 7.95 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1663.9 $[M + H]^+$). ^1H NMR (500 MHz, DMSO-*d*6) δ 8.96 (s, 2H), 8.83 (br s, 4H), 7.80 (s, 2H), 7.51 – 7.48 (m, 8H), 7.47 – 7.41 (m, 4H), 7.39 – 7.35 (m, 4H), 7.28 – 7.19 (m, 6H), 7.15 (t, 2H, *J* = 8.2), 6.39 (br s, 2H), 5.60 (s, 4H), 4.87 (br s, 4H), 4.50 (br s, 4H), 4.44 (t, 4H, *J* = 5.5), 4.30 (q,

4H, *J* = 6.9), 4.05 (br s, 4H), 3.88 (br s, 4H), 3.73 (t, 4H), 3.43 (s, 4H), 3.38 (s, 4H), 3.13 – 3.09 (m, 4H), 1.32 (t, 6H, *J* = 7.1), 1.05 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{84}H_{90}N_{20}O_{13}F_4 + H^+$: 1663.70051, obsd 1663.72310.

Dimeric ligand 27e. Yield after RP-HPLC purification: 20.0 mg (10.3 μmol, 21%). LC-MS analysis: T_R 7.85 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1707.6 $[M + H]^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 8.96 (s, 2H), 8.83 (br s, 4H), 7.73 (s, 2H), 7.51 – 7.07 (m, 24H), 6.20 (br s, 2H, 5.56 (s, 4H, CH_2 $C_6H_4F_2$), 5.10 – 4.65 (br s, 4H), 4.55 – 4.43 (br s, 4H), 4.38 (t, 4H, *J* = 4.5), 4.22 (q, 4H, *J* = 6.9), 4.02 (br s, 4H), 3.88 (br s, 4H), 3.69 (t, 4H, *J* = 5.0), 3.43 – 3.32 (m, 12H), 3.08 – 3.03 (m, 4H), 1.25 (t, 6H, *J* = 7.1), 0.99 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{86}H_{94}N_{20}O_{14}F_4 + H^+$: 1707.72673, obsd 1707.74903.

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Chapter 3

Synthesis and evaluation of homodimeric GnRHR antagonists having a rigid propargylated benzene core[1]

Introduction

The gonadotropin-releasing hormone receptor (GnRHR)[2,3] belongs to the family of membrane bound G-protein coupled receptors (GPCRs). The GnRHR is located in anterior pituitary cells and plays an important role in the reproductive system. Stimulation of the receptor with GnRH, a decapeptidic agonist, initiates the release of the gonadotropins luteinizing hormone (LH)[4] and follicle-stimulating hormone (FSH).[5] FSH and LH in turn induce follicle stimulation and ovulation in females and stimulate steroidogenesis in both males and females. GnRHR antagonists have found widespread use in controlled ovarian stimulation (COS) protocols for IVF treatment. By inhibiting the gonadal axis in males, GnRHR antagonists suppress androgen production, rationalizing their therapeutic use in, for instance, prostate cancer. Several research reports point towards the existence of GnRHR dimers as the active species involved in GnRHR signaling.[6,7] GPCR dimerization and/or oligomerization[8-13] is a well-recognized phenomenon which may be capitalized upon in the development of more active or specific (ant)agonists.[14-17] However, to date there are no dimeric GnRHR ligands known, with the exception of the dimeric

antibody complexed peptide agonist with which the possible relevance of GnRHR dimerization was demonstrated for the first time.[18,19] The results in the preparation and evaluation of a library of homodimeric compounds (A, Figure 1) based on the imidazopyrimidinone GnRHR antagonist **1** were described in Chapter 2.[20] The library was constructed by modification of antagonist **1**[21-23] with an acetylene function and connecting modified ligand **2** to a set of bis-azide functionalized hydrophilic polyethylene glycol spacers by a 1,3-dipolar cycloaddition. As reference compounds, a set of monomeric ligands were prepared in which the bis-azide polyethylene glycol spacers were substituted on one site with the acetylene functionalized ligand. The library of compounds did not contain a dimeric species with either a significantly enhanced GnRHR binding affinity or a significantly enhanced functional (ant)agonistic activity. Although one might conclude from these results that GnRHR dimerization is not a prerequisite for signaling, at least in the Chapter 2 described system, it was considered more likely that the correct dimeric ligand design was not met. Many permutations are possible, and one specific alteration in the design that is addressed here is the replacement of the flexible, hydrophilic polyethylene glycol linker system by the rigid, hydrophobic benzene-based scaffolds as in B depicted in Figure 1. This Chapter addresses the question whether the imposed spatial constraint in the orientation of the pharmacophores exerts an effect on the binding and functional activity of the ligands.

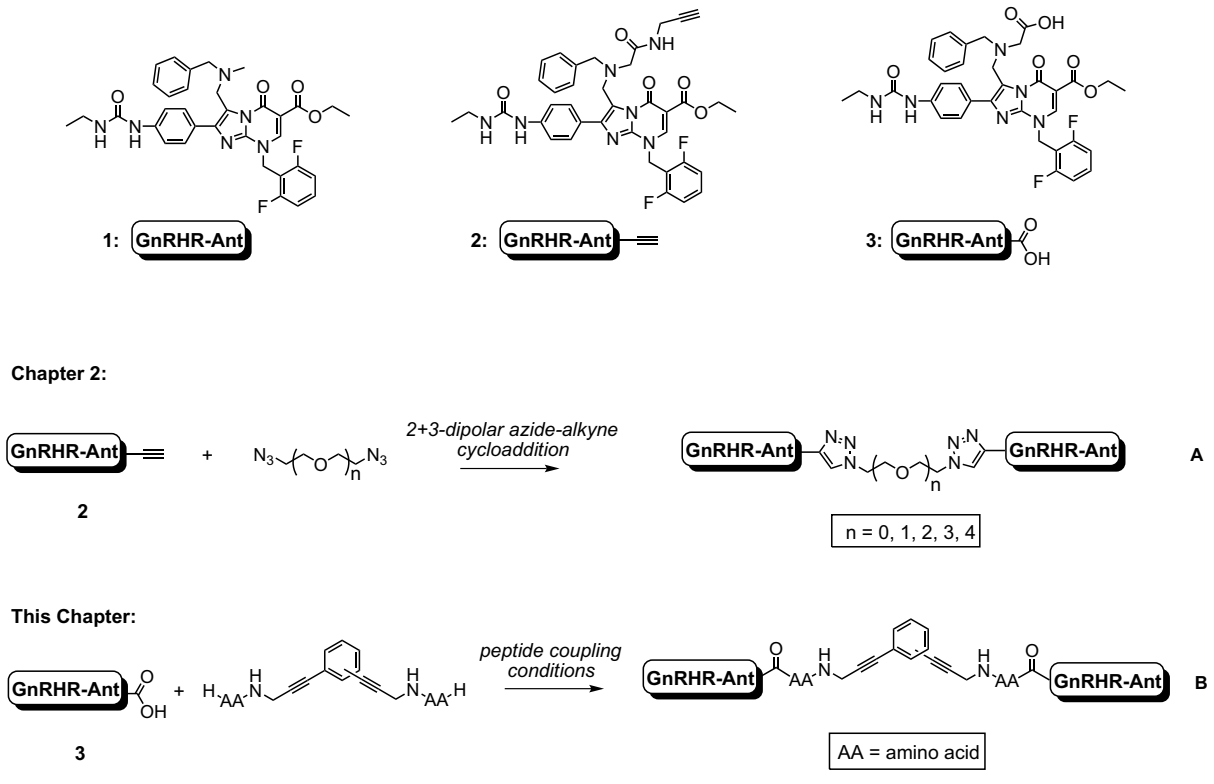
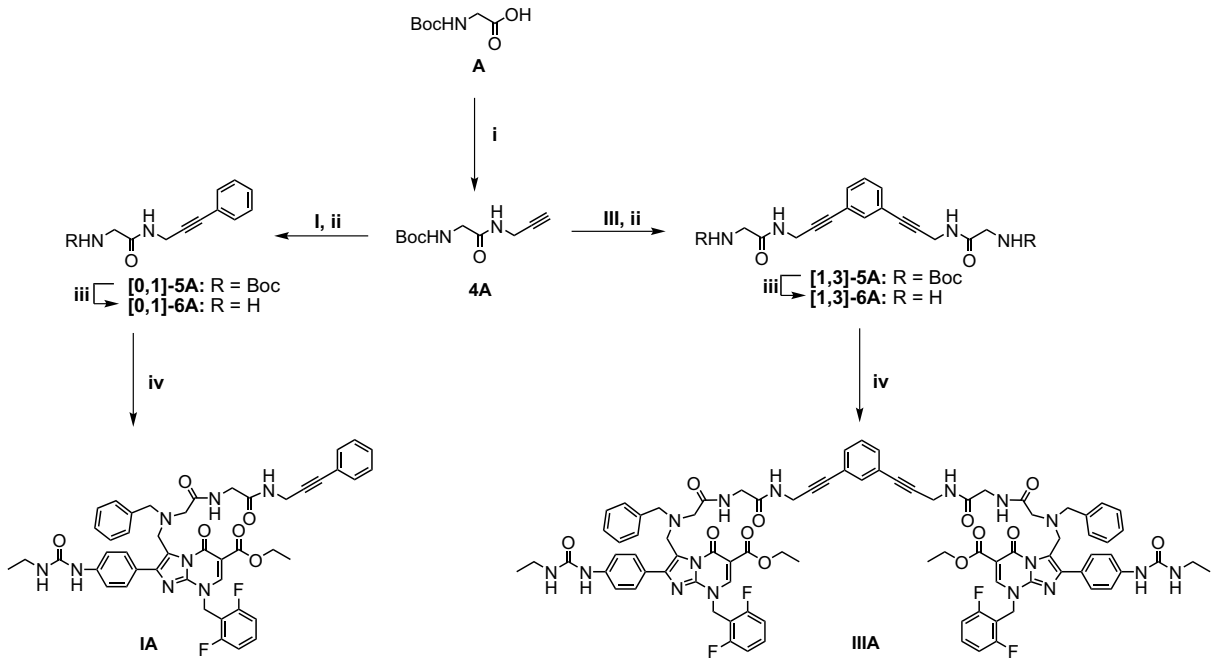


Figure 1. Overview of the in Chapter 2 described library (**A**) and current library (**B**) of dimeric GnRHR antagonists.

Results and discussion

The strategy for the preparation of the second-generation library is outlined in Scheme 1. *N*-Boc-glycine (**A**) was coupled to propargyl amine to afford acetylene **4A** in 76% yield. Sonogashira cross coupling (CuI, $Pd(PPh_3)_4$, pyrrolidine and DMF) of **4A** to phenyl iodide (**I**) gave compound **[0,1]-5A** in 66% yield. After Boc removal (TFA/DCM and 1% TIS) and HPLC purification of the resulting ammonium salt, compound **IA** was prepared by condensation of **[0,1]-6A** with pharmacophore **3** (prepared as described in reference 20) under the agency of BOP and DiPEA in DMF. The HPLC purification of **[0,1]-6A** proved necessary, since direct condensation of crude **[0,1]-6A** after Boc removal with **3** proceeded sluggishly and in low yield. Reaction of **4A** with 1,3-diiodobenzene (**III**) gave bis-propargyl benzene **[1,3]-5A**. Now, Boc-removal, HPLC purification and condensation with 2 equivalents of carboxylate **3** gave bifunctional ligand **IIIA**. In this fashion, a 4 × 7 compound library was assembled, using the four iodo benzene derivatives **I-IV** and the seven amino acids **AG** (Figure 2), leading to seven mono-functional compounds (**IA-G**), seven *ortho*-disubstituted benzene derivatives (**IIA-G**), seven *meta*-disubstituted benzene derivatives (**IIIA-G**) and seven *para*-disubstituted benzene derivatives (**IVA-G**). Synthesis of all members in the library proceeded in an efficiency compared to that of the examples outlined in scheme 1 (see the experimental section for details). All target compounds are depicted in Figure 3.



Scheme 1. Representative route for the synthesis of monomeric and dimeric GnRHR ligands. *Reagents and conditions*: *i.* Isobutyl chloroformate, NMM, propargylamine, DCM, -20 °C to rt 18 h, 76%; *ii.* CuI, $Pd(PPh_3)_4$, pyrrolidine, DMF, 18 h, 66% for **[0,1]-5A**, 99% for **[1,3]-5A**; *iii.* TFA/DCM; 1/1; v/v, 1% TIS, 18 h, HPLC purification; *iv*. pharmacophore **3**, BOP, DiPEA, DMF, 18 h, HPLC purification.

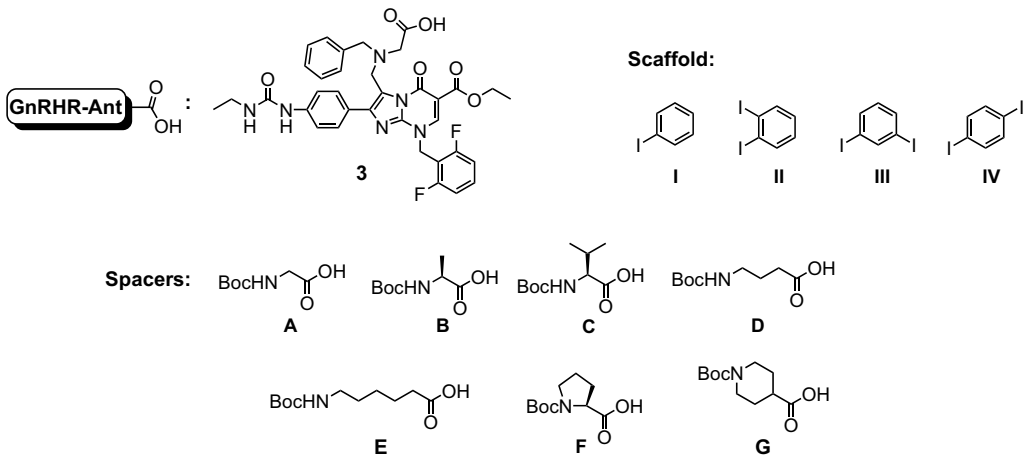


Figure 2. Aryl iodides and amino acids employed in the construction of the library.

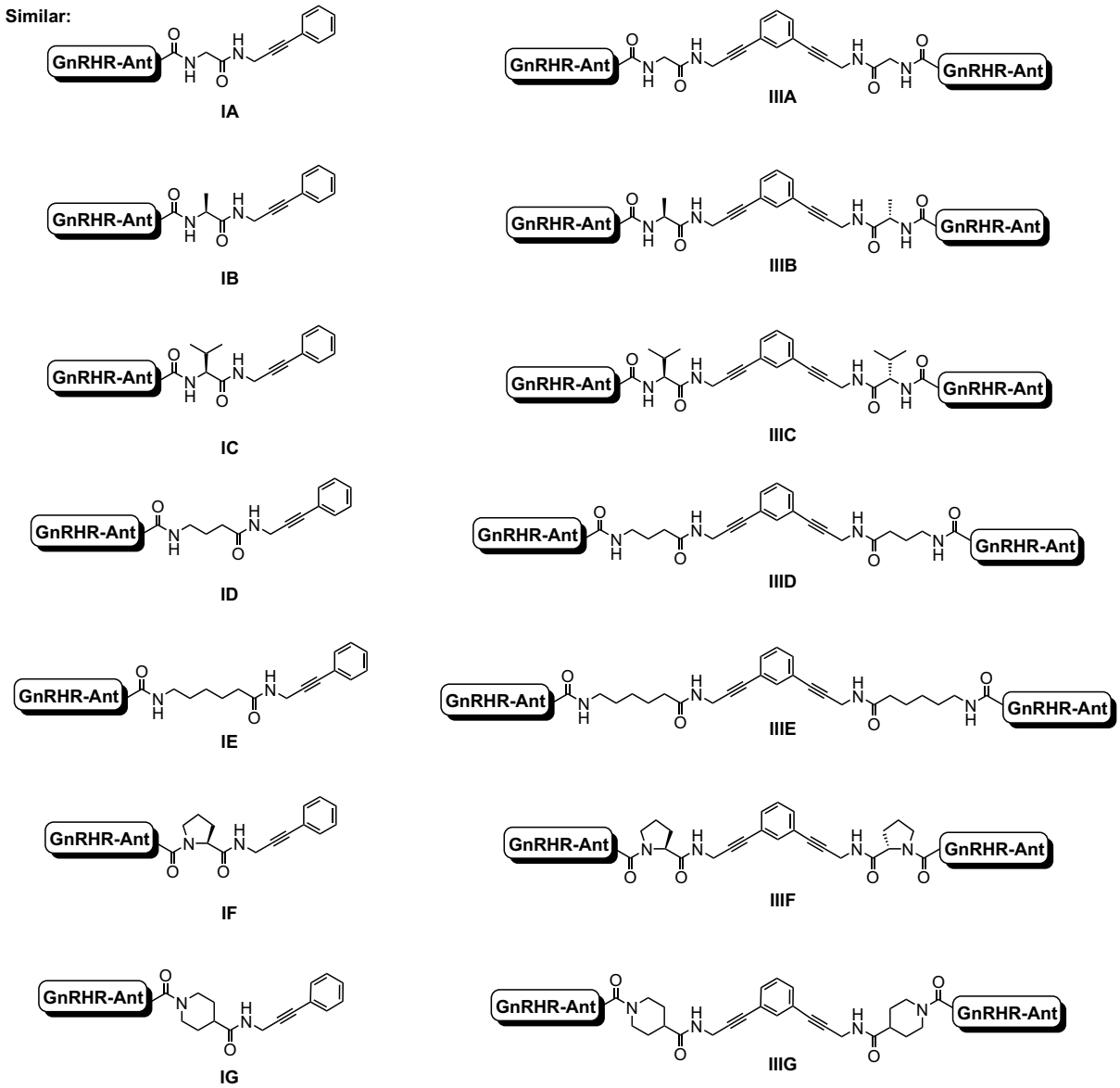


Figure 3. Structures of monovalent ligands **IA-G** and dimeric ligands **IIIA-G**.

All synthesized compounds were tested for their ability to bind to the GnRH receptor and to antagonize GnRH-mediated signaling. The results are summarized in Tables 1 and 2. The binding affinity of the compounds was measured by monitoring their ability to displace the radioactive GnRHR agonist [^{125}I]triptorelin from plasma membranes of GnRHR-expressing Chinese Hamster Ovary (CHO) cells. As is evident from Table 1 most monomeric compounds bind to GnRHR with higher affinity than their corresponding dimeric compounds. An exception is dimeric ligand **IVB** for which a 3-fold increase was observed compared to the monomeric counterpart **IB** ($P<0.05$). In general, the compounds with alanine (**B**) or valine (**C**) spacers possess lower binding affinity compared to the other compounds. Also, the *ortho*- and *meta*- substituted aromatic scaffolds (that is, series **II** and **III** respectively) show slightly reduced affinity compared to the *para*-substituted scaffolds (series **IV**). Exceptions are dimeric ligands with spacer **G**, which show comparable binding affinities in all cases, but reduced affinities compared to the monomeric ligand **IG**.

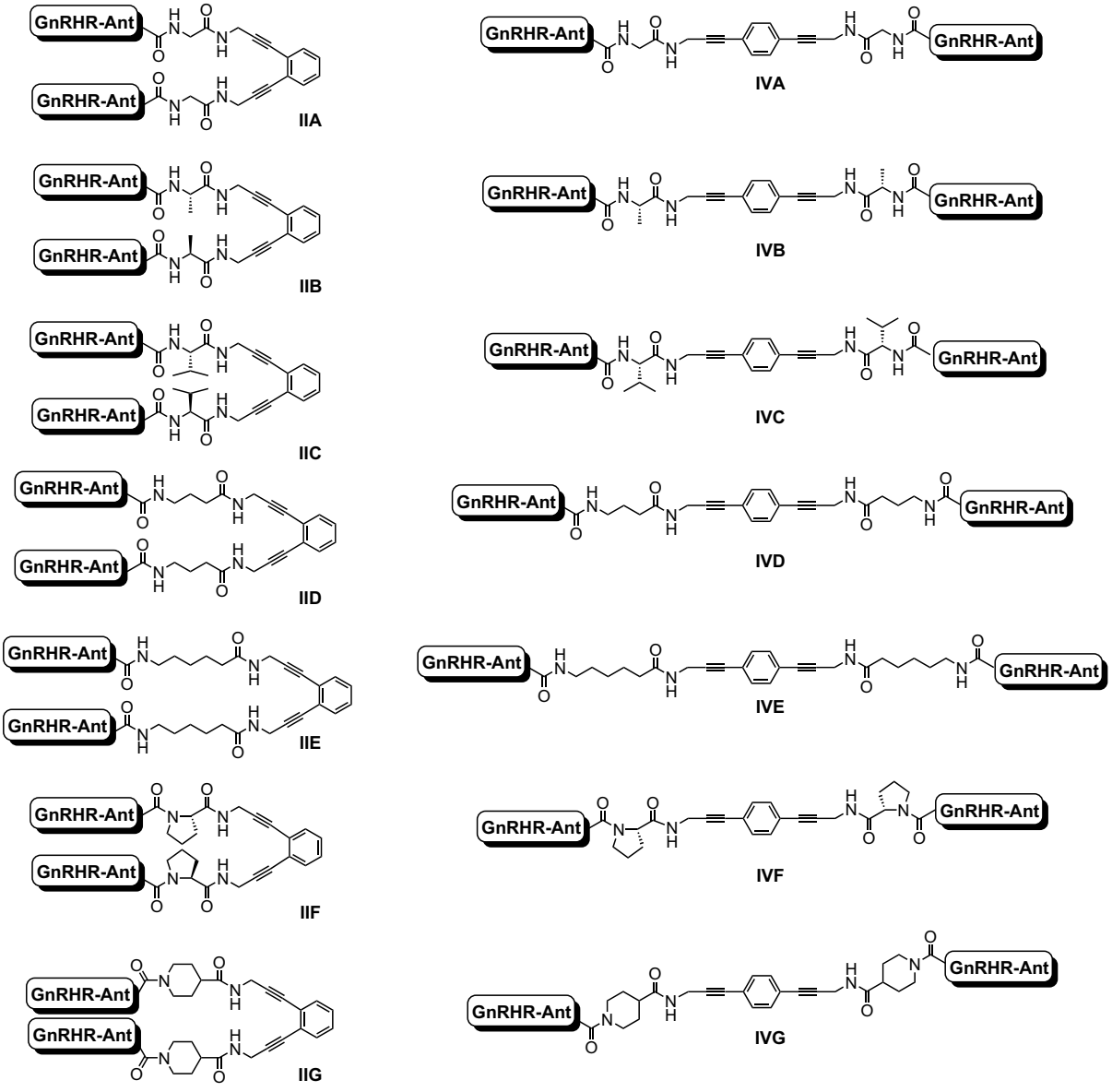


Figure 3 continued. Structures of dimeric ligands **IIA-G**, and **IVA-G**.

		K_i (nM)			
1	4				
	AA	**I**	**II**	**III**	**IV**
A	Gly	166	978	1064	465
B	Ala	1606	2423	5333	474
C	Val	762	3312	2130	714
D	Abu	582	843	818	602
E	Ahx	360	697	934	617
F	Pro	248	851	925	737
G	Pip	131	443	402	514

Table 1. Binding affinities (K_i values) of monomeric GnRHR antagonists **1**, **IA-IG** and dimeric GnRHR antagonists **IIA-G**, **IIIA-G** and **IVA-G.** Abu: aminobutyric acid. Ahx: aminohexanoic acid.Pip: 4-pipecolic acid.

For the functional assay, CHO cells stably transfected with the GnRHR and equipped with the NFAT luciferase reporter gene was used. These cells were stimulated with a submaximal (EC_{80}) concentration of the agonist GnRH in the presence of several concentrations of the test compounds. Antagonistic activity was detected as a decrease of the luminescence signal upon addition of the luciferase substrate. The IC_{50} values of the mono functionalized compounds **IA-G** and the antagonistic effects at 3.16 μM and 10 μM of all compounds (**IA-G**, **IIA-G**, **IIIA-G** and **IVA-G)** are listed in Table 2. The ability of the monomeric ligands to antagonize GnRHR signaling, expressed as IC_{50} values, is in good agreement with the binding affinity (K_i) observed in the radioligand displacement assay (Table 1). However, compounds **IB** and **IC**, the linker system of which is derived from the amino acids alanine (**B**) or valine (**C**) show a reduced effect (E_{max}) compared to the other monomeric ligands. Most of the bifunctional ligands show a concentration dependent inhibition of GnRH-induced luminescence. However, full inhibition at the highest concentration tested (10 μM) was never observed, suggesting that the potency of the dimeric ligands is relatively modest. Six compounds (that is compounds **IID**, **E** and **G**, **IIIG** and **IVD** and **E**) show over 50% antagonism at 3.16 μM. These compounds all hold longer spacers (that is amino butanoate **D**, -hexanoate **E** and pipecolate **G**).

	IC_{50} (nM)								
1	76								
		I		**II**		**III**		**IV**	
	IC_{50} (nM)	% E at 3.16 μM	% E at 10 μM	% E at 3.16 μM	% E at 10 μM	% E at 3.16 μM	% E at 10 μM	% E at 3.16 μM	% E at 10 μM
A	461	92 ± 4	95 ± 3	37 ± 11	73 ± 12	20 ± 9	9 ± 9	34 ± 9	59 ± 6
B	1068	63 ± 5	61 ± 2	13 ± 2	57 ± 7	18 ± 17	14 ± 8	42 ± 7	52 ± 3
C	511	64 ± 9	60 ± 4	0 ± 12	43 ± 13	28 ± 17	43 ± 15	31 ± 0	34 ± 6
D	660	97 ± 5	96 ± 4	73 ± 8	96 ± 3	40 ± 1	57 ± 3	68 ± 15	85 ± 12
E	438	97± 1	99 ± 1	52 ± 12	95 ± 2	44 ± 16	58 ± 13	54 ± 3	67 ± 5
F	556	87 ± 5	89 ± 6	21 ± 7	42 ± 14	31 ± 7	67 ± 8	46 ± 9	65 ± 12
G	463	83 ± 4	82 ± 2	58 ± 6	70 ± 2	68 ± 13	86 ± 12	35 ± 5	48 ± 5

Table 2. Antagonistic activities (IC_{50}) of monomeric GnRHR antagonist **1** and **IA-G** and % inhibition of dimeric GnRHR antagonists **IIA-G**, **IIIA-G** and **IVA-G** at 3.16 and 10 μM concentration. [a]CHO cells that stably express the GnRHR were stimulated with a submaximal (EC_{80}) concentration of GnRH and were incubated with increasing concentrations of the compounds. The IC_{50} value is the concentration of compound needed to inhibit the agonistic response by 50%. The mean IC_{50} are calculated from the -log IC_{50} values from two or three independent experiments performed in duplicate. The SD of pIC_{50} is generally lower than 0.2.

The literature on the antibody-mediated dimerization of GnRHR antagonistic peptides, with agonists as a result,[18] was an incentive to investigate whether the dimeric molecules possess agonistic activity. Additional assays performed with all compounds in an agonistic set-up, that is, when tested alone in the luciferase reporter gene assay, did not provide any actives (data not shown).

The discrepancy in binding and functional properties of the dimeric compounds invited some additional experiments. The effects of two of the ligands were examined on an entire concentration-effect curve of the peptide agonist triptorelin. Thus, triptorelin curves in the absence and in the presence of two concentrations (2 and 10 μM) of selected compounds **ID** and **IVD** was recorded (Figure 4).

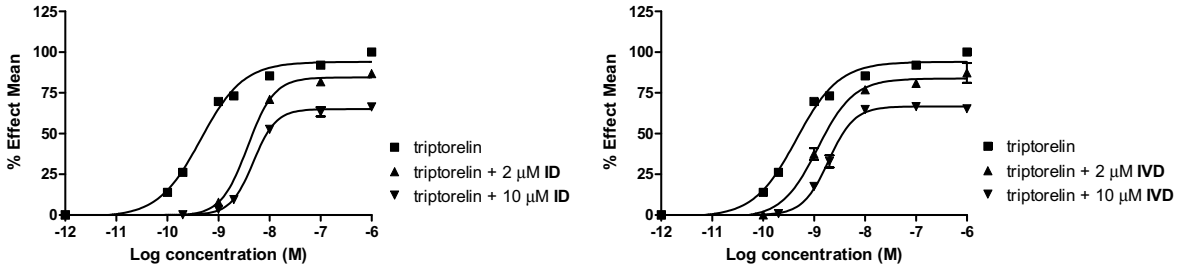


Figure 4. Curve signatures of peptide-agonist triptorelin in the absence and presence of 2 and 10 μM of monomeric ligand **ID** (left) and dimeric ligand **IVD** (right).

As is evident from the curves shown in Figure 4, both monomeric- and dimeric ligand **ID** and **IVD** show a rightward shift of the dose-response curve, a clear indication of the antagonism of the peptide agonist triptorelin. Compound **IVD** shows less antagonistic potency against triptorelin compared to the monomer, which is in agreement with the outcome of the functional assay as depicted in Table 2. However, the maximal efficacy (E_{max}) of triptorelin is also decreased, to the same amount in the presence of both monomeric ligand **ID** and dimeric ligand **IVD**. This result might indicate that the compounds also possess non-competitive characteristics, such as binding to an allosteric binding site that may be close to or partially overlapping with the peptide binding site. In order to exclude the possibility that the reduced efficacy of the compounds is due to cytotoxicity the cell viability by a trypan blue exclusion experiment was determined. It was found that cell viability always exceeded 95 % after 4 h of incubation in the absence (control) or presence of 10 μM of a relevant subset of the compounds (see experimental part).

In this study, dimeric ligands derived from ligand **l** show interesting biological properties compared to the monomeric counterparts. While the binding affinities of the dimeric ligands and monomeric ligands were in the same order of magnitude, some different functional properties were observed for the dimeric ligands. For example, dimeric ligand **IVD** and monomeric ligand **ID** share similar binding affinities in the displacement assay, whereas a decrease in antagonistic potency for dimeric ligand **IVD** is observed. A similar trend was observed for dimeric ligand **IVB**, which show a 3-fold increase in binding affinities compared to monomeric ligand **IB** and a reduced antagonistic potency in the functional assay.
From the functional assay, a general correlation is observed between spacer length and potency of the dimeric ligands. For example, compounds **IID**, **IIE**, **IIG**, **IIIG**, **IVD** and **IVE**, all bearing longer spacers compared to the other compounds, are more potent than the other dimeric compounds. This observation suggests that dimeric ligands in this series with a larger interpharmacophoric distance will exhibit enhanced antagonistic properties.
Additional assays to support evidence for an allosteric interaction of the dimeric ligands to the GnRH receptor show that addition of dimeric ligand **IVD** to the peptide agonist triptorelin reduced both potency and efficacy of the peptide (Figure 4). However, the monomeric counterpart **ID** affected the dose-response curve to the same extent. Recently, a distinct, that is non-GnRH peptide, binding site was reported for a different set of heterocyclic GnRHR antagonists.[24-26] It is conceivable that the compounds bind to the receptor in a similar fashion as the reported non-peptidic antagonists. The dimeric ligands derived from antagonist **1** do not show enhanced pharmacological properties compared to the monomeric counterparts. Therefore, this study does not give new insights into different modes of binding of bivalent ligands to the GnRHR. Recently, in a similar study targeting the serotonin 5-HT_4 receptor dimer specifically with bivalent ligands which are constructed in a similar fashion as the library described in this report, no discrepancy was observed in monomeric and dimeric ligands in respect to binding properties. In the functional assay, most bivalent compounds lost the agonistic character of the monomeric reference compound.[27] Yet, the authors show a conformational change of 5-HT_4R dimers with

bioluminescence resonance energy transfer (BRET) when subjecting the dimeric partial agonist to the receptor thus suggesting a simultaneous interaction of the two pharmacophores of the bivalent ligands to the receptor dimer. The dimeric ligands presented here are highly reminiscent to those reported in the 5-HT_4 study when considering the nature and size of the linker systems. It is therefore not unlikely that the here described dimeric compounds behave in a similar fashion, that is, simultaneous binding to a GnRHR dimer but without a pharmacological effect. However, further studies are required to establish the validity of this hypothesis.

Conclusion

In conclusion, a strategy for the preparation of a set of dimeric ligands containing hydrophobic, rigid linker systems to target the GnRH receptor was developed. The results described in this Chapter concerning binding and functional properties do not provide further information to establish the interaction of dimeric ligands to GnRHR. Combination of the binding and functional antagonistic properties and the reduction of the maximal effect of the peptide agonist triptorelin in the presence of the compounds might indicate a different mode of binding of the dimeric ligands compared to the monomeric counterparts. To establish whether dimerization of GnRH receptor is a prerequisite for signaling or ligand binding, more research is needed. The next Chapter describes the development of a library based on a homo- and heterodimeric ligands containing a different antagonist.

Experimental Procedures

GnRHR Luciferase reporter gene assay

Chinese Hamster Ovary, CHO-K1, cells with stable expression of the human Gonadotropin-Releasing Hormone Receptor (GnRHR) and Nuclear Factor Activated T-cell luciferase reporter gene were grown to 80-90% confluence in culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% w/v Fetal Bovine Serum, 100 units/mL Penicillin and 100 µg/mL Streptomycin and 400 µg/mL Geniticin. On the day of the assay, cells were washed twice with Phosphate Buffered Saline and then harvested with cell dissociation solution. Cells were resuspended in assay medium consisting of DMEM supplemented with 1 mg/L insulin and 5 mg/L apo-transferrin and 3% v/v DMSO. Then, 10 µL cell suspension containing 7,500 cells was added to each well of a 384-well white culture plate. Thereafter, 10 µL of test compound was added at 10 concentrations ranging from final concentration of 10 µM to 0.3 nM with half log intervals. Compounds were allowed to preincubate with cells for 30 min followed by addition of 10 µL agonist GnRH at a final concentration of 3 nM which produces approximately 80% of the maximal effect (EC_{80}) when given alone. After 4 h stimulation, 15 µL of luclite® was added to each well for detection of luciferase protein and plates were left at room temperature for 1 h in the dark. Finally, the luminescence signal was quantified on the TopCount® Microplate Scintillation and Luminescence Counter.

Radioligand Binding Assay.

Ganirelix was provided by Schering-Plough research institute (Oss, The Netherlands). [^{125}I]triptorelin (specific activity 2200 Ci mmol^{-1}) was purchased from Perkin Elmer Life Sciences B.V. (Groningen, The Netherlands). CHO-K1 cells stably expressing the human GnRH receptor were provided by Schering-Plough research institute (Oss, The Netherlands). All other chemicals and cell culture materials were obtained from standard commercial sources.

CHO (Chinese hamster ovary) -K1 cells expressing the wild-type human GnRH receptor were grown in Ham's F12 medium containing 10% bovine calf serum, streptomycin (100 μg mL^{-1}), penicillin (100 IU mL^{-1}) and G418 (0.4 mg mL^{-1}) at 37 °C in 5% CO_2. The cells were subcultured twice weekly at a ratio of 1:20. For membrane preparation the cells were subcultured 1:10 and transferred to large 14-cm diameter plates. For membrane preparation the cells were detached from the plates by scraping them into 5 mL PBS, collected and centrifuged at 1400 *g* (3000 rpm) for 5 min. Pellets derived from 30 plates were pooled and resuspended in 30 mL of ice-cold 50 mM Tris-HCl buffer supplemented with 2 mM $MgCl_2$, pH 7.4. An UltraThurrax was used to homogenize the cell suspension. Membranes and the cytosolic fraction were separated by centrifugation at 100,000 *g* (31,000 rpm) at 4 °C for 20 min. The pellet was resuspended in 10 mL of the Tris-HCl buffer and the homogenization and centrifugation steps were repeated. Tris-HCl buffer (10 mL) was used to resuspend the pellet and the membranes were stored in 500 μL aliquots at -80 °C. Membrane protein concentrations were measured using the BCA (bicinchoninic acid) method.[28]

On the day of the assay membrane aliquots containing 20 μg protein were incubated in a total volume of 100 μL assay buffer (50 mM Tris HCl, pH 7.4, supplemented with 2 mM $MgCl_2$ and 0.1% BSA) at 22 °C for 45 min. Displacement experiments were performed using five concentrations of competing ligand in the presence of 30,000 cpm [^{125}I]triptorelin. Non-specific binding was determined in the presence of 1 μM ganirelix and represented approximately 20% of the total binding. Total binding was determined in the presence of buffer and was set at 100% in all experiments, whereas non-specific binding was set at 0%. Incubations were terminated by dilution with ice-cold Tris HCl buffer. Separation of bound from free radioligand was performed by rapid filtration through Whatman GF/B filters pre-soaked with 0.25 % PEI for 1 h using a Brandel harvester. Filters were subsequently washed three times with ice-cold wash buffer (50 mM Tris HCl, pH 7.4, supplemented with 2 mM $MgCl_2$ and 0.05% BSA). Filter-bound radioactivity was determined in a γ-counter.

All data was analyzed using the non-linear regression curve-fitting program GraphPad Prism v. 4 (GraphPad Software Inc, San Diego, CA, U.S.A.). Inhibitory binding constants (K_i values) were derived from the IC_{50} values according to $K_i = IC_{50}/(1 + [C]/K_d)$ where [C] is the concentration of the radioligand and K_d its dissociation constant.[29] The K_d value (0.35 nM) of [^{125}I]Triptorelin was obtained by computer analysis of saturation curves.[30] All values obtained are means of at least two independent experiments performed in duplicate.

Cytotoxicity

CHOhGnRH_luc cells were seeded on 5-cm diameter plates in assay medium in the absence (control) or presence of 10 μM of test compounds. Compounds **ID-IVD**, **IVA** and **IVE** were selected as relevant compounds in this toxicity assay. The cells were incubated for 4 h at 37 °C. Thereafter the cells were harvested using 0.5 mL trypsol and resuspended in 2 mL of PBS. Subsequently the number of viable cells was determined by trypan blue exclusion, where a trypan blue solution (0.8 % (w/v) in PBS) was added to an equal amount of cell suspension. The proportion of live cells was determined by counting in a hemocytometer.

Chemical procedures.

Reactions were executed at ambient temperatures unless stated otherwise. All moisture sensitive reactions were performed under an argon atmosphere. All solvents were removed by evaporation under reduced pressure. Reactions were monitored by TLC analysis using silica gel coated plates (0.2 mm thickness) and detection by 254 nm UV-light or by either spraying with a solution of $(NH_4)_6Mo_7O_{24} \times 4H_2O$ (25 g/L) or $(NH_4)_4Ce(SO_4)_4 \times 2H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at ~150 °C. Column chromatography was performed on silica gel (40-63 μm). NMR spectra were recorded on a 200/50 MHz, 300/75 MHz, 400/100 MHz, 500/125 MHz or 600/150 MHz spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants (*J*) are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. For LC-MS analysis, a HPLC-system (detection simultaneously at 214 and 254 nm) equipped with an analytical C_{18} column (4.6 mmD × 250 mmL, 5μ particle size) in combination with buffers A: H_2O, B: CH_3CN and C: 1% aq. TFA and coupled to a mass instrument with an electronspray interface (ESI) was used. For RP-HPLC purifications, an automated HPLC system equipped with a preperative C_{18} column (5 μm C_{18}, 10Å, 150 × 21.2 mm) was used. The applied buffers were A: H_2O + 0.1% TFA and B: CH_3CN. High resolution mass spectra were recorded by direct injection (2 μL of a 2 μM solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60000 at *m/z* 400 (mass range *m/z* = 150-2000) and dioctylpthalate (*m/z* = 391.28428) as a lock mass. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan).

General procedure for coupling of amino acids with propargylamine (4A-4G).

Isobutyl chloroformate (1.43 mL, 11 mmol) was added to a cooled (-20 °C) solution of amino acid (10 mmol) and *N*-methylmorpholine (1.52 mL, 14 mmol) in DCM (50 mL). After stirring for 1 h, propargylamine (0.96 mL, 14 mmol) was added. The reaction mixture was warmed to room temperature over a period of 2 h and subsequently stirred for 16 h. The mixture was successively washed with 1M HCl (50 mL) and 10% aqueous $NaHCO_3$ (50 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated. The residue was dissolved in EtOAc and triturated with petroleum ether to afford titled product as white crystals.

***N*-α-*tert*-Boc-glycine propargylamide (4A)**. Yield: 1.60 g (7.55 mmol, 76%). R_f = 0.60 (EtOAc). ESI-MS *m/z*: 213.0 $[M + H]^+$. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.59 (br t, 1H), 5.25 (br t, 1H), 4.07 (dd, 2H, *J* = 5.5 , *J* = 2.6), 3.82 (d, 2H, *J* = 5.8), 2.24 (t, 1H, *J* = 2.6), 1.46 (s, 9H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 169.2, 156.0, 89.8, 80.4, 71.7 (5 × C), 29.1 (CH_2), 28.3 (3 × CH_3).

***N*-α-*tert*-Boc-alanine propargylamide (4B)**. Yield: 1.94 g (8.55 mmol, 86%). R_f = 0.55 (5% MeOH in DCM). ESI-MS *m/z*: 227.0 $[M + H]^+$. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.59 (br t), 5.00 (br d, 1H), 4.28 – 4.12 (m, 1H) 4.05 (dd, 2H, *J* = 5.5 , *J* = 2.6), 2.22 (t, 1H, *J* = 2.6), 1.45 (s, 9H), 1.35 (d, 3H, *J* = 6.9). ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.4, 155.5, 80.2, 79.3, 71.5 (5 × C), 49.9 (CH), 29.1 (CH_2), 28.3 (3 × CH_3), 18.3 (CH_3).

***N*-α-*tert*-Boc-valine propargylamide (4C)**. Yield: 2.52 g (9.92 mmol, 99%). R_f = 0.65 (5% MeOH in DCM). ESI-MS *m/z*: 255.1 $[M + H]^+$. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.40 (br t, 1H), 5.04 (br d, 1H), 4.05 (dd, 2H, *J* = 5.5 , *J* = 2.2), 3.92 (dd, 1H, *J* = 8.9 , *J* = 6.4), 2.22 (t, 1H, *J* = 2.6), 2.15 (m, 1H), 1.45 (s, 9H), 0.97 (d, 3H, *J* = 6.6), 0.93 (d, 3H, *J* = 7.0). ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.6, 155.9, 79.9, 79.3, 71.4 (5 × C), 59.8, 31.0 (2 × CH), 28.9 (CH_2), 28.3 (3 × CH_3), 17.9, 19.1 (2 × CH_3).

***N*-γ-*tert*-Boc-γ-amino butaric acid-propargylamide (4D)**. Yield: 1.68 g (7.00 mmol, 70%). R_f = 0.50 (5% MeOH in DCM). ESI-MS *m/z*: 240.9 $[M + H]^+$. ^{1}H NMR (200 MHz, $CDCl_3$) δ 6.39 (br t, 1H), 4.73 (br t, 1H), 4.05 (dd, 2H, *J* = 5.1 , *J* = 2.9), 3.18 (q, 2H, *J* = 6.6), 2.27 – 2.21 (m, 3H), 1.88 – 1.75 (m, 2H), 1.44 (s, 9H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.2, 156.7, 89.3, 79.3, 70.9 (5 × C), 39.4, 32.9 28.6, (3 × CH_2), 28.1 (3 × CH_3), 25.8 (CH_2).

***N*-ε-*tert*-Boc-ε-amino hexanoic acid-propargylamide (4E)**. Yield: 2.41 g (8.99 mmol, 90%). R_f = 0.60 (5% MeOH in DCM). ESI-MS *m/z*: 269.1 $[M + H]^+$. ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.77 (br t, 1H), 4.56 (br t, 1H), 4.05 (dd, 2H, *J* = 5.5 , *J* = 2.6), 3.11 (q, 2H, *J* = 6.6), 2.24 – 2.17 (m, 3H), 1.44 (s, 9H), 1.74 – 1.34 (m, 6H). ^{13}C NMR (50 MHz, $CDCl_3$/CD_3OD) δ 173.3, 156.3, 79.5, 79.2, 71.0 (5 × C), 39.9, 35.7, 29.3, 28.7 (4 × CH_2), 28.2 (3 × CH_3), 26.0, 24.9 (2 × CH_2).

***N*-α-*tert*-Boc-proline propargylamide (4F)**. Yield: 1.71 g (6.79 mmol, 68%). R_f = 0.55 (5% MeOH in DCM). ESI-MS *m/z*: 253.0 $[M + H]^+$. ^{1}H NMR mixture of rotamers (200 MHz, $CDCl_3$) δ 7.35 (br s, 1H), 6.25 (br s, 1H), 4.26 (br s, 1H), 4.02 (br s, 2H), 3.35 (br s, 2H), 2.23 (s, 1H), 2.13 (br s, 2H), 1.88 (br s, 2H), 1.46 (br s, 9H). ^{13}C NMR of rotamers (50 MHz, $CDCl_3$) δ 171.9, 80.3, 79.3, 71.2 (4 × C), 46.8, 28.7 (2 × CH_2), 28.3 (3 × CH_3), 24.2, 23.6 (2 × CH_2).

***N*-δ-*tert*-Boc-4-amino cyclohexanoic acid-α-propargylamide (4G)**. Yield: 0.97 g (3.65 mmol, 37%). R_f = 0.60 (5% MeOH in DCM). ESI-MS *m/z*: 267.0 $[M + H]^+$. ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.65 (s, 1H), 4.25 – 4.02 (m, 2H), 4.05 (dd, 2H, *J* = 5.1 , *J* = 2.6), 2.74 (br t, 2H, *J* = 11.0), 2.24 (t, 1H, *J* = 2.6), 2.33 – 2.18 (m, 1H), 1.85 – 1.73 (m, 2H), 1.73 – 1.58 (m, 2H), 1.45 (s, 9H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.1, 154.5, 79.5, 71.3 (4 × C), 43.0 (CH_2), 42.6 (CH), 28.9, 28.3 (2 × CH_2), 28.3 (3 × CH_3).

General procedure for coupling of propargylamide functionalized amino acids with iodobenzene (affording [0,1]-5A-5G).

In separate flasks, a solution of the propargyl functionalized amino acid (**4A-G**, 1.0 mmol), iodobenzene (1.5 mmol, 111μL) in pyrrolidine/DMF (1/4; v/v), a solution of CuI (0.1 mmol, 19.1 mg) in DMF (2 mL) and a solution of $Pd(PPh_3)_4$ (0.05 mmol, 57.8 mg) in DMF (4 mL) were flushed with argon for 1 h in an ultrasonic bath. To the alkyne solution were added subsequently the CuI and the $Pd(PPh_3)_4$ solutions and the mixture were stirred for 18 h under inert atmosphere. The volatiles were removed and the crude product dissolved in MeOH/DCM (1/9; v/v, 50 mL) and washed with water (3 × 50 mL) and 10% aqueous $NaHCO_3$ (50 mL). The organic layer was dried with Na_2SO_4, filtrated and concentrated. The crude material was purified by automated silica gel column chromatography (35 to 65% MeOH/DCM (1/10) in Petroleum ether).

***N*-α-*tert*-Boc-(3-phenylprop-2-yn-1-amide)-glycine ([0,1]-5A)**. Yield: 189 mg (0.66 mmol, 66%). R_f = 0.65 (EtOAc). ESI-MS *m/z*: 289.0 $[M + H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39 (m, 2H), 7.28 (m, 3H), 7.17 (br t, 1H), 5.69 (s, 1H), 4.28 (d, 2H, *J* = 4.5), 3.88 (s, 2H), 1.43 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.5, 156.1 (2 × C), 131.5, 128.1 (2 × CH), 122.3 (C), 84.4, 83.1 (2 × C), 80.1 (C), 43.9, 29.7 (2 × CH_2), 28.0 (3 × CH_3).

***N*-α-*tert*-Boc-(3-phenylprop-2-yn-1-amide)-alanine ([0,1]-5B)**. Yield: 298 mg (0.99 mmol, 99%). R_f = 0.70 (EtOAc). ESI-MS *m/z*: 303.0 $[M + H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39 (m, 2H), 7.27 (m, 3H), 7.09 (s, 1H), 5.42 (d, 1H, *J* = 7.0), 4.30 (br s, 1H), 4.26 (d, 2H, *J* = 4.5), 1.40 (s, 9H), 1.37 (d, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.5, 155.5 (2 × C), 131.5, 128.1 (2 × CH), 122.5, 84.6, 83.1, 79.9 (4 × C), 49.8 (CH), 29.7 (CH_2), 28.2 (3 × CH_3), 18.3 (CH_3).

***N*-α-*tert*-Boc-(3-phenylprop-2-yn-1-amide)-valine ([0,1]-5C)**. Yield: 322 mg (0.98 mmol, 98%). R_f = 0.80 (EtOAc). ESI-MS *m/z*: 331.0 $[M + H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.56 (s, 1H), 7.37 (m, 2H), 7.27 (m, 3H), 5.64 (d, 1H, *J* = 9.5), 4.34 (dd, 1H, *J* = 17.5 , *J* = 5.5), 4.13 (d, 2H, *J* = 4.5), 2.10 – 2.06 (m, 1H), 1.42 (s, 9H), 0.99 (d, 3H, *J* = 7.0), 0.96 (d, 3H, *J* = 7.0). ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.8 (C), 155.9 (C), 131.5, 128.0, (2 × CH), 122.5, 84.7, 82.8, 79.5 (4 × C), 59.6, 31.2 (2 × CH), 29.4 (CH_2), 28.2 (3 × CH_3), 19.0, 18.1 (2 × CH_3).

***N*-α-*tert*-Boc-(3-phenylprop-2-yn-1-amide)-amino butaric acid ([0,1]-5D)**. Yield: 205 mg (0.65 mmol, 65%). R_f = 0.55 (EtOAc). ESI-MS *m/z*: 317.0 $[M + H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41 (m, 2H), 7.29 (m, 3H), 7.23 (s, 1H), 5.25 (s, 1H), 4.23 (s, 2H), 3.13 (t, 2H, *J* = 4.0), 2.25 (t, 2H, *J* = 7.0), 1.83 – 1.78 (m, 2H), 1.43 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.9, 155.6 (2 × C), 131.5, 128.2 (2 × CH), 122.5, 84.7, 82.9, 79.3 (4 × C), 39.5, 29.6, 25.9, 33.1 (4 × CH_2), 28.2 (3 × CH_3).

***N*-α-*tert*-Boc-(3-phenylprop-2-yn-1-amide)-amino hexanoic acid ([0,1]-5E)**. Yield: 332 mg (0.97 mmol, 97%). R_f = 0.70 (EtOAc). ESI-MS *m/z*: 343.1 $[M + H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38 (m, 2H), 7.28 (m, 3H), 7.15 (s, 1H), 5.12 (s, 1H), 4.23 (d, 2H, *J* = 4.0), 3.06 (t, 2H, *J* = 4.0), 2.22 (t, 2H, *J* = 7.5), 1.65 (m, 2H), 1.49 (m, 2H), 1.43 (s, 9H), 1.32 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.3, 155.3 (2 × C), 131.4, 128.0 (2 × CH), 122.4, 84.7, 82.7, 79.0 (4 × C), 39.9, 35.7, 29.5, 29.2 (4 × CH_2), 28.1 (3 × CH_3), 25.9, 24.9 (2 × CH_2).

***N*-α-*tert*-Boc-(3-phenylprop-2-yn-1-amide)-proline ([0,1]-5F)**. Yield: 302 mg (0.92 mmol, 92%). R_f = 0.55 (EtOAc). ESI-MS *m/z*: 329.1 $[M + H]^+$.. ^{1}H NMR of rotamers (500 MHz, $CDCl_3$) δ 7.39 (m, 2H), 7.27 (m, 3H), 6.63 (br s, 1H), 4.35 (br d, 1H), 4.24 (br s, 2H), 3.40 (br d, 2H), 2.25 (br dd, 2H), 1.84 (br d, 2H), 1.45 (s, 9H). ^{13}C NMR of rotamers (125 MHz, $CDCl_3$) δ 172.2, 155.3 (2 × C), 131.4, 128.0 (2 × CH), 122.3, 84.7, 82.9, 80.2 (4 × C), 60.8 (CH), 46.8, 29.5 (2 × CH_2), 28.1 (3 × CH_3), 24.4, 23.1 (2 × CH_2).

***N*-α-*tert*-Boc-(3-phenylprop-2-yn-1-amide)-amino cyclohexanoic acid ([0,1]-5G)**. Yield: 323 mg (0.94 mmol, 94%). R_f = 0.85 (EtOAc). ESI-MS *m/z*: 343.0 $[M + H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39 (m, 2H), 7.28 (m, 3H), 6.43 (s, 1H), 4.26 (d, 2H, *J* = 5.0), 4.12 (br s, 2H), 2.72 (br s, 2H), 2.30 (m, 1H), 1.80 (m, 2H), 1.65 (m, 2H), 1.45 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.1, 154.5 (2 × C) 131.5, 128.1 (2 × CH), 122.3, 84.7, 83.1, 79.5 (4 × C), 42.9 (CH_2), 42.8 (CH), 29.8, 28.3 (2 × CH_2), 27.4 (3 × CH_3).

General procedure for coupling of propargylic spacers 4A-G with 1,2-diiodobenzene (affording [1,2]-5A-G), 1,3-diiodobenzene (affording [1,3]-5A-G) and 1,4-diiodobenzene (affording [1,4]-5A-G).

In separate flasks, a solution of the propargyl functionalized amino acid (**4A-G**, 0.90 mmol), the desired diiodobenzene (0.30 mmol, 91.1 mg) and pyrrolidine (1.80 mmol, 147 µL) in DMF (3 mL), a solution of CuI (0.06 mmol, 11.5 mg) in DMF (1 mL) and a solution of $Pd(PPh_3)_4$ (0.03 mmol, 34.7 mg) in DMF (1 mL) were was flushed with argon for 1 h in an ultrasonic bath. To the alkyne solution were added subsequently the CuI and the $Pd(PPh_3)_4$ solutions and the mixture were stirred for 18 h under argon atmosphere. The volatiles were removed and the crude product dissolved in MeOH/DCM (1/9, v/v, 50 mL) and washed with water (3 × 10 mL) and 10% aqueous $NaHCO_3$ (10 mL). The organic layer was dried with Na_2SO_4 and concentrated. The crude material was purified by automated silica gel column chromatography (35 to 65% MeOH/DCM (1/10) in Petroleum ether).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,2-phenylene)diprop-2-yn-1-amide)-glycine ([1,2]-5A)**. Yield: 148 mg (0.30 mmol, 99%). R_f = 0.40 (EtOAc). ESI-MS *m/z*: 443.9 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (br s, 2H), 7.37 – 7.34 (m, 2H), 7.24 – 7.20 (m, 2H), 5.97 (br t, 2H), 4.29 (d, 4H, *J* = 5.4), 3.88 (d, 4H, *J* = 5.2), 1.44 (s, 18H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 170.0, 156.2 (2 × C), 131.5, 127.9 (2 × CH), 125.4, 88.9, 81.2, 79.9 (4 × C), 44.0, 29.8 (2 × CH_2), 28.2 (3 × CH_3).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,2-phenylene)diprop-2-yn-1-amide)-alanine ([1,2]-5B)**. Yield: 157 mg (0.3 mmol, 99%). R_f = 0.75 (EtOAc). ESI-MS *m/z*: 527.4 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 (br s, 2H), 7.39 – 7.34 (m, 2H), 7.24 – 7.20 (m, 2H), 5.67 (d, 2H, *J* = 7.3), 4.37 (d, 4H, *J* = 5.1), 4.30 – 4.00 (m, 2H), 1.41 (s, 18H), 1.37 (t, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.3, 155.7 (2 × C), 131.6, 127.9 (2 × CH), 125.6, 88.9, 81.3, 79.9 (4 × C), 49.9 (CH), 29.9 (CH_2), 28.2 (3 × CH_3), 18.4 (CH_3).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,2-phenylene)diprop-2-yn-1-amide)-valine ([1,2]-5C)**. Yield: 164 mg (0.28 mmol, 94%). R_f = 0.75 (50% EtOAc in toluene). ESI-MS *m/z*: 583.4 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (br s, 2H), 7.37 – 7.34 (m, 2H), 7.22 – 7.20 (m, 2H), 5.64 (br s, 2H), 4.48 (dd, 2H, *J* = 17.6 , *J* = 5.8), 4.13 – 4.02 (m, 4H), 2.09 – 2.01 (m, 2H), 1.41 (s, 18H), 0.93 (d, 12H, *J* = 6.8). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.4, 156.1 (2 × C), 131.5, 127.7 (2 × CH), 125.5, 88.9, 80.9, 79.5 (4 × C), 60.0, 31.0 (2 × CH), 29.6 (CH_2), 28.2 (3 × CH_3), 19.2, 18.4 (2 × CH_3).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,2-phenylene)diprop-2-yn-1-amide)-amino butaric acid ([1,2]-5D)**. Yield: 118 mg (0.21 mmol, 71%). R_f = 0.35 (EtOAc). ESI-MS *m/z*: 555.4 $[M + H]^+$. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.69 (s, 2H), 7.40 – 7.20 (m, 4H), 5.18 (s, 2H), 4.27 (d, 4H, *J* = 5.5), 3.18 – 3.12 (m, 4H), 2.38 – 2.24 (m, 4H), 1.90 – 1.74 (m, 4H), 1.43 (s, 18H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 173.1, 156.4 (2 × C), 131.5, 127.9 (2 × CH), 125.5, 89.3, 81.0, 79.0 (4 × C), 39.6, 33.2, 29.8 (3 × CH_2), 28.3 (3 × CH_3), 26.2 (CH_2).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,2-phenylene)diprop-2-yn-1-amide)-amino hexanoic acid ([1,2]-5E)**. Yield: 181 mg (0.30 mmol, 99%). R_f = 0.43 (10% MeOH in DCM). ESI-MS *m/z*: 611.4 $[M + H]^+$. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.73 (br t, 2H), 7.40 – 7.20 (m, 4H), 5.21 (br t, 2H), 4.04 (d, 4H, *J* = 5.1), 3.06 (t, 4H, *J* = 5.5), 2.30 – 2.15 (m, 4H), 1.72 – 1.60 (m, 4H), 1.43 (s, 18H), 1.43 – 1.23 (m, 8H). ^{13}C NMR (50 MHz, $CDCl_3$/CD_3OD) δ 173.6, 156.3 (2 × C), 131.5, 127.8 (2 × CH), 125.3, 89.0, 80.8, 79.0 (4 × C), 39.9, 35.6, 29.4, 29.2 (4 × CH_2), 28.1 (3 × CH_3), 25.9, 25.0 (2 × CH_2).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,2-phenylene)diprop-2-yn-1-amide)-proline ([1,2]-5F)**. Yield: 170 mg (0.29 mmol, 98%). R_f = 0.35 (EtOAc). ESI-MS *m/z*: 579.4 $[M + H]^+$. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.39 – 7.20 (m, 4H), 7.05 (br s, 2H), 6.50 (br s, 2H), 4.35 – 4.20 (m, 4H), 4.20 – 4.05 (m, 2H), 3.56 – 3.30 (m, 4H), 2.40 – 2.05 (m, 4H), 2.05 – 1.82 (m, 4H), 1.42 (s, 18H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.5, 131.6 (2 × CH), 125.4, 89.0, 80.5, 80.3 (4 × C), 60.0 (CH), 46.9, 29.7, 29.3 (3 × CH_2), 28.2 (3 × CH_3), 23.9 (CH_2).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,2-phenylene)diprop-2-yn-1-amide)-amino cyclohexanoic acid ([1,2]-5G)**. Yield: 144 mg (0.24 mmol, 79%). R_f = 0.70 (EtOAc). ESI-MS *m/z*: 607.3 $[M + H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.67 – 7.62 (m, 2H), 7.50 – 7.46 (m, 2H), 4.25 (d, 4H, *J* = 5.4), 4.23 – 4.03 (m, 4H), 2.85 – 2.62 (m, 4H), 2.55 – 2.37 (m, 2H), 1.85 – 1.61 (m, 8H), 1.45 (s, 18H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.7, 154.5 (2 × C), 131.9, 128.3 (2 × CH), 125.6, 89.5, 80.9, 79.3 (4 × C), 43.1 (CH_2), 40.6 (CH), 29.6 (CH_2), 28.2 (3 × CH_3), 27.7 (CH_2).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,3-phenylene)diprop-2-yn-1-amide)-glycine ([1,3]-5A)**. Yield: 148 mg (0.30 mmol, 99%). R_f = 0.50 (EtOAc). ESI-MS *m/z*: 499.5 $[M + H]^+$. ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.45 – 7.16 (m, 4H), 7.07 (br t, 2H), 5.57 (br t, 2H), 4.25 (d, 4H, *J* = 5.1), 3.86 (d, 4H, *J* = 5.1), 1.44 (s, 18H). ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.0, 156.2 (2 × C), 131.5, 127.9 (2 × CH), 125.4, 88.9, 81.2, 79.9 (4 × C), 44.0, 29.6 (2 × CH_2), 28.2 (3 ×

CH_3).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,3-phenylene)diprop-2-yn-1-amide)-alanine ([1,3]-5B)**. Yield: 128 mg (0.24 mmol, 81%). R_f = 0.70 (EtOAc). ESI-MS *m/z*: 527.4 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.68 (br s, 2H), 7.65 – 7.20 (m, 4H), 5.61 (br d, 2H), 4.41 – 4.29 (m, 2H), 4.24 (d, 4H, *J* = 4.4), 1.43 (s, 18H), 1.37 (d, 6H, *J* = 6.9). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.8, 155.5 (2 × C), 131.9, 128.4 (2 × CH), 122.7, 85.4, 82.0, 79.8 (4 × C), 49.8 (CH), 29.7 (CH_2), 28.2 (3 × CH_3), 18.4 (CH_3).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,3-phenylene)diprop-2-yn-1-amide)-valine ([1,3]-5C)**. Yield: 179 mg (0.30 mmol, 99%). R_f = 0.83 (10% MeOH in DCM). ESI-MS *m/z*: 605.6 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.18 (m, 4H), 7.02 (br s, 2H), 5.36 (br d, 2H), 4.24 (d, 4H, *J* = 4.4), 4.06 – 3.98 (m, 2H), 2.18 – 2.06 (m, 2H), 1.43 (s, 18H), 0.99 – 0.94 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.8, 156.0 (2 × C), 134.8, 131.3, 128.2 (3 × CH), 122.9, 85.3, 82.4, 79.8 (4 × C), 59.9 (CH), 31.0 (CH_3), 29.7 (CH_2), 28.3 (3 × CH_3), 19.2 (CH).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,3-phenylene)diprop-2-yn-1-amide)-amino butaric acid ([1,3]-5D)**. Yield: 165 mg (0.30 mmol, 99%). R_f = 0.30 (EtOAc). ESI-MS *m/z*: 555.3 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.69 – 7.21 (m, 4H), 6.77 (br s, 2H), 4.84 (br s, 2H), 4.25 (d, 4H, *J* = 5.2), 3.21 – 3.09 (m, 4H), 2.35 – 2.20 (m, 4H), 1.88 – 1.75 (m, 4H), 1.44 (s, 18H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.7, 156.4 (C), 134.7, 131.8, 128.4 (3 × CH), 122.8, 85.6, 81.9, 79.1 (4 × C), 39.7, 33.2, 29.7 (CH_2), 28.3 (3 × CH_3), 26.1 (CH_2).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,3-phenylene)diprop-2-yn-1-amide)-amino hexanoic acid ([1,3]-5E)**. Yield: 179 mg (0.29 mmol, 98%). R_f = 0.45 (EtOAc). ESI-MS *m/z*: 611.4 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.42 – 7.19 (m, 4H), 7.01 (t, 2H, *J* = 5.2), 4.89 (br t, 2H), 4.24 (d, 4H, *J* = 5.2), 3.12 – 3.02 (m, 4H), 2.28 – 2.19 (m, 6H), 1.70 – 1.58 (m, 6H), 1.52 – 1.40 (m, 22H), 1.38 – 1.27 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.7, 156.0 (2 × C), 134.7, 131.3, 128.3 (3 × CH), 122.8, 85.9, 81.9, 78.9 (4 × C), 40.2, 36.0, 29.6 (3 × CH_2), 28.3 (3 × CH_3), 26.2, 25.1 (2 × CH_2).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,3-phenylene)diprop-2-yn-1-amide)-proline ([1,3]-5F)**. Yield: 172 mg (0.30 mmol, 99%). R_f = 0.45 (EtOAc). ESI-MS *m/z*: 579.2 $[M + H]^+$. ^1H NMR (300 MHz, $CDCl_3$) δ 7.37 – 7.15 (m, 4H), 6.43 (br s, 1H), 4.19 (m, 6H), 3.42 (m, 4H), 2.15 (m, 4H), 1.83 (m, 4H), 1.41 (s, 9H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.8, 155.8 (2 × C), 134.6, 131.4, 128.1 (3 × CH), 122.7, 85.4, 82.1,
80.5 (4 × C), 60.5 (CH), 46.7, 30.5, 29.3 (3 × CH_2), 28.2 (3 × CH_3), 24.0 (CH_2).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,3-phenylene)diprop-2-yn-1-amide)-amino cyclohexanoic acid ([1,3]-5G)**. Yield: 133 mg (0.22 mmol, 73%). R_f = 0.55 (EtOAc). ESI-MS *m/z*: 607.4 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.40 (s, 1H), 7.31 – 7.20 (m, 3H), 6.87 (t, 2H, *J* = 2.4), 4.21 (d, 4H, *J* = 5.2), 4.20 – 4.02 (m, 4H), 2.80 – 2.65 (m, 4H), 2.40 – 2.30 (m, 2H), 1.88 – 1.73 (m, 4H), 1.72 – 1.58 (m, 4H), 1.41 (s, 18H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.2, 154.6 (2 × C), 143.8, 131.4, 128.3 (3 × CH), 122.8, 85.7, 82.1, 79.6 (4 × C), 43.1, 42.8, 29.7, 28.4 (4 × CH_2), 28.3 (3 × CH_3).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,4-phenylene)diprop-2-yn-1-amide)-glycine ([1,4]-5A)**. Yield: 148 mg (0.30 mmol, 99%). R_f = 0.70 (EtOAc). ESI-MS *m/z*: 499.5 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.25 (s, 4H), 7.09 (br t, 2H), 5.71 (br t, 2H), 4.24 (d, 4H, *J* = 5.2), 3.85 (s, 4H), 1.44 (s, 18H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.5, 156.2 (2 × C), 131.5 (4 × CH), 122.5, 86.5, 82.6, 80.2 (4 × C), 44.2, 29.7 (2 × CH_2), 28.3 (3 × CH_3).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,4-phenylene)diprop-2-yn-1-amide)-alanine ([1,4]-5B)**. Yield: 91.5 mg (0.17 mmol, 58%). R_f = 0.70 (EtOAc). ESI-MS *m/z*: 527.2 $[M + H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.42 (s, 2H), 7.29 (s, 4H), 5.58 (br d, 2H), 4.31 – 4.15 (m, 6H), 1.43 (s, 18H), 1.38 (d, 6H, *J* = 7.2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.4, 155.6 (2 × C), 131.5 (4 × CH), 122.5, 86.5, 82.7, 80.2 (4 × C), 49.9 (CH), 29.8 (CH_2), 28.2 (3 × CH_3), 18.2 (CH_3).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,4-phenylene)diprop-2-yn-1-amide)-valine ([1,4]-5C)**. Yield: 140 mg (0.23 mmol, 77%). R_f = 0.75 (10% MeOH in DCM). ESI-MS *m/z*: 605.6 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 (s, 4H), 6.70 (s, 2H), 5.26 (br d, 2H), 4.32 – 4.19 (m, 4H), 4.04 – 3.96 (m, 2H), 2.20 – 2.02 (m, 2H), 1.44 (s, 18H), 1.01 – 0.90 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.5, 156.0 (2 × C), 131.5 (4 × CH), 122.5, 86.4, 82.8, 79.9 (4 × C), 59.9 (CH), 30.9 (CH_3), 29.8 (CH_2), 28.2 (3 × CH_3), 19.2 (CH).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,4-phenylene)diprop-2-yn-1-amide)-amino butaric acid ([1,4]-5D)**. Yield: 135 mg (0.23 mmol, 78%). R_f = 0.45 (EtOAc). ESI-MS *m/z*: 577.4 $[M + Na]^+$. ^{1}H NMR (400 MHz, DMSO-*d*6) δ 7.38 (s, 4H), 6.80 (br s, 2H), 4.10 (d, 4H, *J* = 5.2), 2.93 – 2.89 (m, 4H), 2.12 – 2.05 (m, 4H), 1.66 – 1.53 (m, 4H), 1.36 (s, 18H). ^{13}C NMR (150 MHz, $CDCl_3$) δ 172.9, 156.8 (2 × C), 132.8 (4 × CH), 123.6, 90.4, 82.2, 78.7 (4 × C), 56.1, 33.8, 29.8 (3 × CH_2), 29.5 (3 × CH_3), 26.9 (CH_2).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,3-phenylene)diprop-2-yn-1-amide)-amino hexanoic acid ([1,4]-5E)**. Yield: 64 mg (0.11, 35%). R_f = 0.40 (EtOAc). ESI-MS *m/z*: 611.3 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-*d*6) δ 8.30 (t, 2H, *J* = 5.2), 7.37 (s, 4H), 5.75 (t, 2H, *J* = 2.4), 4.09 (t, 4H, *J* = 2.8), 2.86 (q, 4H, *J* = 6.8), 2.07 (t, 4H, *J* = 7.6), 1.51 – 1.43 (m, 4H,), 1.35 (s, 18H), 1.36 – 1.31 (m, 4H), 1.23 – 1.16 (m, 4H). ^{13}C NMR (150 MHz, DMSO-*d*6) δ 171.9, 155.5 (2 × C), 131.5 (4 × CH), 122.2, 89.2, 80.9, 77.2 (4 × C), 35.0, 29.2, 28.5 (3 × CH_2), 28.2 (3 × CH_3), 25.9, 24.8 (2 × CH_2).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,3-phenylene)diprop-2-yn-1-amide)-proline ([1,4]-5F)**. Yield: 172 mg (0.30 mmol, 99%). R_f = 0.40 (EtOAc). ESI-MS *m/z*: 579.1 $[M + H]^+$. ^{1}H NMR (400 MHz, DMSO-*d*6) δ 8.42 (t, 2H, *J* = 2.8), 7.35 (m, 4H), 4.12 (d, 4H, *J* = 5.6), 4.05 – 4.02 (m, 2H), 3.43 – 3.31 (m, 4H), 3.30 – 3.20 (m, 2H), 2.17 – 2.01 (m, 2H), 1.85 – 1.68 (m, 4H), 1.30 (s, 18H). ^{13}C NMR (100 MHz, DMSO-*d*6) δ 172.4, 153.2 (2 × C), 131.5 (4 × CH), 122.3, 90.2, 80.8, 78.4 (4 × C), 59.7 (CH), 46.4, 30.3, 28.4 (3 × CH_2), 27.9 (3 × CH_3), 23.6 (CH_2).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,3-phenylene)diprop-2-yn-1-amide)-amino cyclohexanoic acid ([1,4]-5G)**. Yield: 120 mg (0.20 mmol, 66%). R_f = 0.45 (EtOAc). ESI-MS *m/z*: 607.5 $[M + H]^+$. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.28 (s, 4H), 6.19 (t, 2H, *J* = 4.9), 4.24 (d, 4H, *J* = 5.1), 4.13 – 4.08 (m, 4H), 2.70 (t, 4H, *J* = 12.0), 2.31 – 2.22 (m, 2H), 1.81 – 1.77 (m, 4H), 1.69 – 1.55 (m, 4H), 1.42 (s, 18H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 174.0, 154.5 (2 × C), 131.5 (4 × CH), 122.5, 86.7, 82.7, 79.6 (4 × C), 43.0, 42.9, 29.8, 28.4 (4 × CH_2), 28.3 (3 × CH_3).

General procedure for coupling of pharmacophore 3 with monomeric spacers [0,1]-5A-G affording IA-G.

Boc protected compounds **[0,1]-5A-G** were subjected to a solution of 1/1 DCM/TFA v/v + 1% TIS for 18 h. The volatiles were evaporated and the compounds were purified with semi-preparative HPLC system (0 to 40% B). Accordingly, 20 μmol of amine (**[0,1]-6A-G**) was dissolved in 100 μL of DMF and added to a solution containing pharmacophore **3**[20] (30 μmol, 13.4 mg), BOP (39 μmol, 17.6 mg) and DiPEA (120 μmol, 20.4 μL) in 300 μL DMF. The reaction mixture was stirred at rt for 16 h and diluted with a mixture of DCM and MeOH (9/1; v/v, 20 mL). The organic layer was successively washed with water (3 × 10 mL), 10% aqueous $NaHCO_3$ (3 × 10 mL) and brine (1

× 20 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated. The crude product was purified on a semi-preparative HPLC system (40 to 60% B) and lyophilized to obtain the title compounds as amorphous solids.

Monomeric ligand IA. Yield after RP-HPLC purification: 3.0 mg (3.6 µmol, 12%). LC-MS analysis: t_R 7.00 min (gradient 30 to 90% B) and t_R 3.75 min (gradient 50 to 90% B). ESI-MS *m/z*: 843.5 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.82 (s, 1H), 8.54 (s, 1H), 8.36 (t, 1H, *J* = 5.5), 7.93 (t, 1H, *J* = 5.7), 7.61 (d, 2H, *J* = 8.4), 7.47 – 7.33 (m, 9H), 7.21 – 7.06 (m, 6H), 6.15 (t, 1H, *J* = 5.5), 5.54 (s, 2H), 5.31 (t, 1H, *J* = 5.2), 4.26 (m, 4H), 4.10 (d, 2H, *J* = 5.4), 3.63 – 3.61 (m, 4H), 3.32 – 3.30 (m, 2H), 3.13 – 3.09 (m, 2H), 1.30 (t, 3H, *J* = 7.1), 1.08 (t, 3H, *J* = 7.2). HRMS *m/z* calcd for $C_{46}H_{44}F_2N_8O_6 + H^+$: 843.34246, obsd 843.34254.

Monomeric ligand IB. Yield after RP-HPLC purification: 4.3 mg (5.0 µmol, 17%). LC-MS analysis: t_R 7.37 min (gradient 30 to 90% B) and t_R 4.23 min (gradient 50 to 90% B). ESI-MS *m/z*: 856.9 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.82 (s, 1H), 8.53 (s, 1H), 8.37 (t, 1H, *J* = 5.3), 7.86 (d, 1H, *J* = 7.8), 7.57 (d, 2H, *J* = 8.4), 7.48 – 7.42 (m, 4H), 7.35 – 7.30 (m, 5H), 7.28 – 7.05 (m, 6H), 6.68 (br s, 1H), 5.54 (s, 2H), 5.31 (t, 1H, *J* = 5.1), 4.36 (d, 1H, *J* = 13.2), 4.29 – 4.22 (m, 4H), 4.08 (d, 2H, *J* = 1.9), 3.68 – 3.53 (m, 4H), 3.12 – 3.06 (m, 2H), 1.33 (t, 3H, *J* = 7.1), 1.25 – 1.20 (m, 3H), 1.03 (t, 3H, *J* = 7.2). HRMS *m/z* calcd for $C_{47}H_{46}F_2N_8O_6 + H^+$: 857.35811, obsd 857.35817.

Monomeric ligand IC. Yield after RP-HPLC purification: 5.1 mg (5.8 µmol, 19%). LC-MS analysis: t_R 8.02 min (gradient 30 to 90% B) and t_R 4.82 min (gradient 50 to 90% B). ESI-MS *m/z*: 885.6 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.80 (s, 1H), 8.66 (s, 1H), 8.45 (t, 1H, *J* = 5.4 Hz), 7.88 (m, 1H), 7.60 (d, 2H, *J* = 9.0), 7.46 – 7.07 (m, 15H), 6.27 (s, 1H), 5.54 (s, 2H), 5.31 (t, 1H, *J* = 4.9), 4.38 (d, 1H, *J* = 13.2), 4.28 – 4.20 (m, 3H), 4.14 – 4.03 (m, 4H), 3.69 – 3.58 (m, 4H), 3.20 (d, 2H, *J* = 16.2), 3.11 – 3.08 (m, 2H), 1.85 – 1.83 (m, 1H), 1.30 (t, 3H, *J* = 7.1), 1.05 (t, 3H, *J* = 7.2), 0.72 (d, 3H, *J* = 6.6), 0.65 (d, 3H, *J* = 6.6). HRMS *m/z* calcd for $C_{49}H_{50}F_2N_8O_6 + H^+$: 885.38941, obsd 885.38886.

Monomeric ligand ID. Yield after RP-HPLC purification: 5.8 mg (6.7 µmol, 22%). LC-MS analysis: t_R 7.15 min (gradient 30 to 90% B) and t_R 3.71 min (gradient 50 to 90% B). ESI-MS *m/z*: 871.7 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.79 (s, 1H), 8.84 (s, 1H), 8.27 (t, 1H, *J* = 3.6), 7.64 (m, 1H, *J* = 3.6), 7.59 (d, 2H, *J* = 8.4), 7.45 (d, 2H, *J* = 8.4), 7.39 – 7.28 (m, 7H), 7.19 – 7.07 (m, 6H), 6.49 (br s, 1H), 5.53 (s, 2H), 5.31 (t, 1H, *J* = 4.8), 4.32 – 4.26 (m, 4H), 4.10 – 4.07 (m, 2H), 3.54 (s, 2H), 3.12 – 3.00 (m, 4H), 2.90 (q, 2H, *J* = 6.0), 2.13 – 2.05 (m, 2H), 1.52 – 1.49 (m, 2H), 1.31 (t, 3H, *J* = 7.2), 1.05 (t, 3H, *J* = 7.2). HRMS *m/z* calcd for $C_{48}H_{48}F_2N_8O_6$, + H^+: 871.37376, obsd 871.37354.

Monomeric ligand IE. Yield after RP-HPLC purification: 5.3 mg (5.9 µmol, 20%). LC-MS analysis: t_R 7.52 min (gradient 30 to 90% B) and t_R 4.42 min (gradient 50 to 90% B). ESI-MS *m/z*: 899.0 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.80 (s, 1H), 8.55 (s, 1H), 8.27 (t, 1H, *J* = 3.6), 6.85 (br s, 1H), 7.59 (d, 2H, *J* = 8.4), 7.44 (d, 2H, *J* = 8.4), 7.39 – 7.32 (m, 7H), 7.18 – 7.06 (m, 6H), 6.15 (t, 1H, *J* = 6.0), 5.53 (s, 2H), 5.31 (t, 1H, *J* = 5.4), 4.33 (s, 2H), 4.28 (q, 2H, *J* = 7.2), 4.09 – 4.07 (m, 2H), 3.54 (s, 2H), 3.13 – 3.00 (m, 4H), 2.79 (q, 2H, *J* = 6.6), 2.09 – 2.02 (m, 2H), 1.50 – 1.39 (m, 2H), 1.30 – 1.13 (m, 7H), 1.06 (t, 3H, *J* = 7.2). HRMS *m/z* calcd for $C_{50}H_{52}F_2N_8O_6 + H^+$: 899.40506, obsd 899.40559.

Monomeric ligand IF. Yield after RP-HPLC purification: 7.03 mg (8.0 µmol, 27%). LC-MS analysis: t_R 7.56 min (gradient 30 to 90% B) and t_R 4.21 min (gradient 50 to 90% B). ESI-MS *m/z*: 883.4 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.81 (s, 1H), 8.64 (s, 1H), 8.39 (t, 1H, *J* = 3.6), 7.60 (d, 2H, *J* = 9.0), 7.45 – 7.03 (m, 15H), 6.30 (t, 1H, *J* = 6.0), 5.56 (s, 2H), 5.31 (t, 1H, *J* = 4.8), 4.38 (br d, 1H), 4.30 – 4.25 (m, 2H), 4.12 – 4.04 (m, 4H), 3.70 – 3.59

(m, 4H), 3.12 – 3.06 (m, 4H), 1.90 – 1.85 (m, 1H), 1.72 – 1.65 (m, 1H), 1.62 – 1.52 (m, 2H), 1.29 (t, 3H, *J* = 7.2), 1.04 (t, 3H, *J* = 7.2). HRMS *m/z* calcd for $C_{49}H_{48}F_2N_8O_6 + H^+$: 883.37376, obsd 883.37334.

Monomeric ligand IG. Yield after RP-HPLC purification: 2.0 mg (2.2 μmol, 7%). LC-MS analysis: t_R 7.41 min (gradient 30 to 90% B) and t_R 4.27 min (gradient 50 to 90% B). ESI-MS *m/z*: 897.7 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d6*) δ 8.84 (s, 1H), 8.51 (s, 1H), 8.27 (t, 1H, *J* = 3.6), 7.59 (d, 2H, *J* = 9.0), 7.43 – 7.35 (m, 8H), 7.21 – 7.12 (m, 7H), 6.15 (t, 1H, *J* = 3.6), 5.55 (s, 2H), 5.31 (t, 1H, *J* = 5.0), 4.38 – 4.26 (m, 4H), 4.12 – 3.98 (m, 3H), 3.62 – 3.55 (m, 3H), 3.30 – 3.23 (m, 1H), 3.14 – 3.08 (m, 3H), 2.52 – 2.49 (m, 1H), 2.23 – 2.20 (m, 2H), 1.55 – 1.52 (m, 1H), 1.30 (t, 3H, *J* = 7.2), 1.29 – 1.17 (m, 3H), 1.05 (t, 3H, *J* = 7.2). HRMS *m/z* calcd for $C_{50}H_{50}F_2N_8O_6 + H^+$: 897.38941, obsd 897.38932.

***General procedure for coupling of pharmacophore 3 with dimeric spacers* [1,2]-5A-G, [1,3]-5A-G *or* [1,4]-5A-G *affording IIIA-G, IIA-G or IVA-G*.**

Boc protected compounds **[1,2]-5A-G**, **[1,3]-5A-G**or **[1,4]-5A-G** were subjected to a solution of 1/1 DCM/TFA v/v + 1% TIS for 18 h. The volatiles were evaporated and the compounds were purified with semi-preparative HPLC system (0 to 30% B). Accordingly, 15 μmol of amine (**[1,2]-6A-G**, **[1,3]-6A-G** or **[1,4]-6A-G**) was dissolved in 100 μL of DMF and added to a solution containing pharmacophore **3**[20] (33 μmol, 14.8 mg), BOP (39 μmol, 17.6 mg) and DiPEA (120 μmol, 20.4 μL) in 300 μL DMF. The reaction mixture was stirred at rt for 16 h and diluted with a mixture of DCM and MeOH (9/1; v/v, 20 mL). The organic layer was successively washed with water (3 × 10 mL), 10% aqueous $NaHCO_3$ (3 × 10 mL) and brine (1 × 20 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated. The crude product was purified on a semi-preparative HPLC system (40 to 60% B) and lyophilized to obtain the title compounds as amorphous solids.

Dimeric ligand IIA. Yield after RP-HPLC purification: 1.83 mg (1.1 μmol, 8%). LC-MS analysis: t_R 8.43 min (gradient 30 to 90% B) and t_R 5.74 min (gradient 50 – 90% B). ESI-MS *m/z*: 1607.8 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d6*) δ 8.81 (s, 2H), 8.56 (s, 2H), 8.36 (br t, 2H), 7.92 (br t, 2H), 7.63 –7.55 (m, 4H), 7.52 – 7.28 (m, 12H), 7.21 – 7.02 (m, 12H), 6.17 (s, 2), 5.53 (s, 4H), 5.31 (t, 2H, *J* = 4.8), 4.32 – 4.22 (m, 8H), 4.18 – 4.12 (m, 4H), 3.59 – 3.56 (m, 8H), 3.18 – 3.08 (m, 8H), 1.29 (t, 6H, *J* = 7.0), 1.05 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{86}H_{82}F_4N_{16}O_{12} + 2H^+$: 804.31899, obsd 804.31909.

Dimeric ligand IIB. Yield after RP-HPLC purification: 2.09 mg (1.3 μmol, 9%). LC-MS analysis: t_R 8.80 min (gradient 30 to 90% B) and t_R 6.25 min (gradient 50 to 90% B). ESI-MS *m/z*: 1636.9 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d6*) δ 8.79 (s, 2H), 8.55 (s, 2H), 8.32 (br s, 2H), 7.86 (br s, 2H), 7.62 – 7.28 (m, 16H), 7.19 – 7.02 (m, 12H), 6.16 (br s, 2H), 5.53 (s, 4H), 5.31 (t, 2H, *J* = 4.8), 4.36 – 4.30 (m, 2H), 4.28 – 4.22 (m, 8H), 4.18 – 4.07 (m, 4H), 3.66 – 3.55 (m, 4H), 3.18 – 3.05 (m, 8H), 1.32 – 1.27 (m, 12H), 1.05 (m, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{88}H_{86}F_4N_{16}O_{12} + 2H^+$: 816.33464, obsd 818.33529.

Dimeric ligand IIC. Yield after RP-HPLC purification: 1.50 mg (0.9 μmol, 6%). LC-MS analysis: t_R 9.54 min (gradient 30 to 90% B) and t_R 7.38 min (gradient 50 to 90% B). ESI-MS *m/z*: 1693.1 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d6*) δ 8.80 (s, 2H), 8.66 (s, 2H), 8.45 (br s, 2H), 7.78 (br s, 2H), 7.60 (d, 4H, *J* = 9.0), 7.53 – 7.05 (m, 24H), 6.12 (s, 2H), 5.54 (s, 4H), 5.31 (t, 2H, *J* = 5.4), 4.38 (br d, 2H), 4.31 – 4.02 (m, 12), 3.68 – 3.55 (m, 4H), 3.17 – 3.05 (m, 8H), 1.89 – 1.82 (m, 2H), 1.28 (t, 6H, *J* = 7.2), 0.97 (t, 6H, *J* = 7.2), 0.73 (br d, 6H), 0.65 (br d, 6H). HRMS *m/z* calcd for $C_{92}H_{94}F_4N_{16}O_{12} + 2H^+$: 846.36594, obsd 846.36597.

Dimeric ligand IID. Yield after RP-HPLC purification: 1.26 mg (0.8 µmol, 5%). LC-MS analysis: t_R 8.57 min (gradient 30 to 90% B) and t_R 5.88 min (gradient 50 to 90% B). ESI-MS *m/z*: 1663.9 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.79 (s, 2H), 8.52 (s, 2H), 8.27 (br t, 2H), 7.68 – 7.58 (m, 6H, 7.48 – 7.38 (m, 6H), 7.35 – 7.30 (m, 2H), 7.20 – 7.12 (m, 10H), 7.08 – 7.01 (m, 6H), 6.15 (br t, 2H), 5.52 (s, 4H), 5.31 (t, 2H, *J* = 5.4), 4.32 – 4.26 (m, 7H), 4.15 – 4.10 (m, 5H), 3.56 – 3.49 (m, 4H), 3.15 – 3.02 (m, 8H), 2.93– 2.88 (m, 4H), 1.97 – 1.90 (m, 4H), 1.57 – 1.50 (m, 4H), 1.28 (t, 6H, *J* = 7.8), 1.05 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{90}H_{90}F_4N_{16}O_{12} + 2H^+$: 832.35029, obsd 832.35010.

Dimeric ligand IIE. Yield after RP-HPLC purification: 1.42 mg (0.8 µmol, 6%). LC-MS analysis: t_R 8.89 min (gradient 30 to 90% B) and t_R 6.48 min (gradient 50 to 90% B). ESI-MS *m/z*: 1721.1 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.80 (s, 2H), 8.55 (s, 2H), 8.30 (br s, 2H), 7.80 (br s, 2H), 7.59 (br d, 4H), 7.50 – 7.48 (m, 8H), 7.37 – 7.30 (m, 2H), 7.38 – 7.03 (m, 14H), 6.15 (br t, 2H), 5.53 (s, 4H), 5.31 (t, 2H, *J* = 5.4), 4.37 – 4.22 (m, 8H), 4.16 – 4.11 (m, 4H), 3.60 – 3.49 (br s, 4H), 3.15 – 2.97 (m, 8H), 2.82 – 2.73 (m, 4H), 2.12 – 2.05 (m, 4H), 1.48 – 1.40 (m, 4H), 1.30 – 1.14 (m, 14H), 1.05 (t, 6H, *J* = 6.6). HRMS *m/z* calcd for $C_{94}H_{98}F_4N_{16}O_{12} + 2H^+$: 860.38159, obsd 860.38187.

Dimeric ligand IIF. Yield after RP-HPLC purification: 1.55 mg (0.9 µmol, 6%). LC-MS analysis: t_R 9.76 min (gradient 30 to 90% B) and t_R 7.51 min (gradient 50 to 90% B). ESI-MS *m/z*: 1687.4 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.78 (s, 2H), 8.52 (s, 2H), 8.28 (t, 2H), 7.62 (d, 1H, *J* = 8.4), 7.57 (d, 4H, *J* = 8.4), 7.48 – 7.39 (m, 6H), 7.35 (d, 4H, *J* = 8.4), 7.30 – 7.19 (m, 6H), 7.18 – 7.05 (m, 4H), 7.03 – 6.95 (m, 3H), 6.15 (t, 2H, *J* = 5.4), 5.53 (s, 4H), 5.31 (t, 2H), 4.35 – 4.28 (m. 2H), 4.24 (q, 4H, *J* = 6.6), 4.15 – 4.02 (m, 5H), 3.67 – 3.40 (m, 8H), 3.15 – 3.05 (m, 4H), 2.99 (s, 4H), 1.92 – 1.80 (m, 2H), 1.72 – 1.63 (m, 2H), 1.62 – 1.42 (m, 4H), 1.28 (m, 6H, *J* = 6.6), 1.04 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{92}H_{90}F_4N_{16}O_{12} + 2H^+$: 844.35029, obsd 844.34997.

Dimeric ligand IIG. Yield after RP-HPLC purification: 2.87 mg (1.7 µmol, 11%). LC-MS analysis: t_R 9.72 min (gradient 30 to 90% B) and t_R 7.56 min (gradient 50 to 90% B). ESI-MS *m/z*: 1716.1 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.81 (s, 2H), 8.49 (s, 2H), 8.27 (t, 2H, *J* = 3.6), 7.59 (d, 4H, *J* = 9.0), 7.50 – 7.39 (m, 4H), 7.38 – 7.32 (m, 6H), 7.20 – 7.07 (m, 14H), 6.13 (t, 2H, *J* = 5.4), 5.55 (s, 4H), 4.34 – 4.24 (m, 8H), 4.13 – 4.08 (m, 4H), 4.03 (br d, 2H), 3.66 – 3.58 (m, 6H), 3.28 – 3.25 (m, 1H), 3.17 – 3.07 (m, 3H), 2.56 – 2.50 (m, 2H), 2.28 – 2.17 (m, 4H), 2.53 (br d, 2H), 1.29 (t, 6H, *J* = 7.2), 1.28 – 1.20 (m, 6H), 1.05 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{94}H_{94}F_4N_{16}O_{12} + 2H^+$: 858.36594, obsd 858.36542.

Dimeric ligand IIIA. Yield after RP-HPLC purification: 3.32 mg (2.1 µmol, 14%). LC-MS analysis: t_R 8.02 min (gradient 30 to 90% B) and t_R 4.90 min (gradient 50 to 90% B). ESI-MS *m/z*: 1607.8 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.81 (s, 2H), 8.56 (s, 2H), 8.36 (br t, 2H), 7.97 – 7.90 (m, 2H), 7.61 (d, 4H, *J* = 8.4), 7.46 – 7.32 (m, 12H), 7.20 – 7.15 (m, 12H), 6.17 (br t, 2H), 5.53 (s, 4H), 5.31 (t, 2H, *J* = 3.6), 4.27 – 4.25 (m, 8H), 4.17 – 4.08 (m, 4H), 3.66 – 3.58 (m, 8H), 3.18 – 3.04 (m, 8H), 1.30 (t, 6H, *J* = 7.2), 1.05 (t, 6H, *J* = 7.1). HRMS *m/z* calcd for $C_{86}H_{82}F_4N_{16}O_{12} + 2H^+$: 804.31899, obsd 804.31900.

Dimeric ligand IIIB. Yield after RP-HPLC purification: 1.13 mg (0.7 µmol, 5%). LC-MS analysis: t_R 8.62 min (gradient 30 to 90% B) and t_R 5.71 min (gradient 50 to 90% B). ESI-MS *m/z*: 1636.9 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.84 (s, 2H), 8.51 (s, 2H), 8.29 (t, 2H, *J* = 4.8), 7.59 (d, 4H, *J* = 9.0), 7.43 – 7.35 (m, 12H), 7.21 – 7.12 (m, 12H), 6.15 (t, 2H, *J* = 4.8), 5.55 (s, 4H,), 5.31 (t, 2H, *J* = 5.10), 4.40 –4.25 (m, 10H), 4.12 – 3.99 (d, 3H), 3.68 – 3.55 (m, 4H), 3.30 – 3.20 (m, 4H), 3.16 – 3.07 (m, 4H), 1.32 – 28 (m, 12H), 1.05 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{88}H_{86}F_4N_{16}O_{12} + 2H^+$: 818.33464, obsd 818.33554.

Dimeric ligand IIIC. Yield after RP-HPLC purification: 3.56 mg (2.1 μmol, 14%). LC-MS analysis: t_R 9.61 min (gradient 30 to 90% B) and t_R 7.04 min (gradient 50 to 90% B). ESI-MS *m/z*: 1693.1 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.82 (s, 2H), 8.55 (s, 2H), 8.49 (br t, 2H), 7.75 (d, 2H, *J* = 9.0), 7.60 (d, 4H, *J* = 9.0), 7.48 – 7.40 (m, 4H), 7.39 (d, 4H, *J* = 9.0), 7.36 – 7.07 (m, 16H), 6.14 (br t, 2H), 5.54 (s, 4H), 5.31 (t, 2H, *J* = 3.6), 4.37 (br d, 2H), 4.30 – 4.05 (m, 8H), 3.62 (dd, 4H, *J* = 63.6 , *J* = 19.8), 3.20 (d, 4H, *J* = 16.8), 3.15 – 3.04 (m, 8H), 1.87 – 1.78 (m, 2H), 1.29 (t, 6H, *J* = 7.1), 1.04 (t, 6H, *J* = 7.1), 0.71 (d, 6H, *J* = 6.6), 0.63 (d, 6H, *J* = 6.6). HRMS *m/z* calcd for $C_{92}H_{94}F_4N_{16}O_{12} + 2H^+$: 846.36594, obsd 846.36585.

Dimeric ligand IIID. Yield after RP-HPLC purification: 3.35 mg (2.0 μmol, 14%). LC-MS analysis: t_R 8.08 min (gradient 30 to 90% B) and t_R 5.01 min (gradient 50 to 90% B). ESI-MS *m/z*: 1665.0 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.81 (s, 2H), 8.57 (s, 2H), 8.29 (br t, 2H), 7.67 (br t, 2H), 7.60 (d, 4H, *J* = 9.0), 7.50 – 7.30 (m, 16H), 7.25 – 7.05 (m, 12H), 6.19 (br t, 2H), 5.53 (s, 4H), 5.31 (br t, 2H), 4.36 – 4.28 (m, 7H), 4.16 – 4.04 (m, 5H), 3.60 – 3.49 (m, 4H), 3.15 – 3.05 (m, 8H), 2.92 – 2.76 (m. 4), 2.18 – 2.12 (m, 4H), 1.56 – 1.48 (m, 4H), 1.30 (t, 6H, *J* = 7.08), 1.04 (t, 6H, *J* = 7.14). HRMS *m/z* calcd for $C_{90}H_{90}F_4N_{16}O_{12} + 2H^+$: 832.35029, obsd 832.34990.

Dimeric ligand IIIE. Yield after RP-HPLC purification: 2.53 mg (1.5 μmol, 10%). LC-MS analysis: t_R 8.61 min (gradient 30 to 90% B) and t_R 5.78 min (gradient 50 to 90% B). ESI-MS *m/z*: 1720.1 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.80 (s, 2H), 8.54 (s, 2H), 8.30 (br t, 2H), 8.26 (br t, 2H), 7.58 (d, 4H, *J* = 8.4), 7.50 – 7.28 (m, 12H), 7.22 – 7.20 (m, 16H), 6.16 (br t, 2H), 5.53 (s, 4H), 5.31 (br t, 2H), 4.33 (s, 4H), 4.27 (q, 4H, *J* = 6.6), 4.12 – 4.00 (m, 4H), 3.58 – 3.50 (m, 4H), 3.15 – 2.98 (m, 8H), 2.82 – 2.75 (m, 4H), 2.25 – 1.94 (m, 4H), 1.52 – 1.38 (m, 4H), 1.30 (t, 6H, *J* = 6.6), 1.22 – 1.12 (m, 8H), 1.05 (t, 6H, *J* = 7.1). HRMS *m/z* calcd for $C_{94}H_{98}F_4N_{16}O_{12} + 2H^+$: 860.38159, obsd 860.38145.

Dimeric ligand IIIF. Yield after RP-HPLC purification: 1.59 mg (0.9 μmol, 6%). LC-MS analysis: t_R 8.93 min (gradient 30 to 90% B) and t_R 6.43 min (gradient 50 to 90% B). ESI-MS *m/z*: 1689.0 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.82 (s, 2H), 8.60 (s, 2H), 8.35 (br s, 2H), 7.90 (m, 2H), 7.60 (d, 4H, *J* = 9.0), 7.49 – 7.06 (m, 24H), 6.21 (br t, 2H), 5.56 (s, 4H), 5.31 (t, 2H, *J* = 4.8), 4.38 (br d, 2H), 4.34 – 4.22 (m, 4H), 4.12 – 4.00 (m, 5H), 3.69 – 3.60 (m, 4H), 3.56 (s, 4H), 3.16 – 3.05 (m, 4H), 1.95 – 1.52 (m, 8H), 1.28 (t, 6H, *J* = 7.2), 1.04 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{92}H_{90}F_4N_{16}O_{12} + 2H^+$: 844.35029, obsd 844.35087.

Dimeric ligand IIIG. Yield after RP-HPLC purification: 2.57 mg (1.5 μmol, 10%). LC-MS analysis: t_R 8.50 min (gradient 30 to 90% B) and t_R 6.03 min (gradient 50 to 90% B). ESI-MS *m/z*: 1717.1 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.82 (s, 2H), 8.69 (s, 2H), 8.26 (s, 2H), 7.59 (d, 4H, *J* = 8.4), 7.51 – 7.35 (m, 12H), 7.25 – 7.06 (m, 12H), 6.15 (t, 2H, *J* = 5.4), 5.55 (s, 4H), 5.31 (br t, 2H), 4.37 – 4.22 (m, 8H), 4.15 – 3.97 (m, 6H), 3.67 – 3.56 (m, 6H), 3.28 – 3.23 (m, 2H), 2.58 – 2.50 (m, 2H), 2.29 – 2.12 (m, 4H), 1.78 – 1.70 (m, 2H), 1.58 – 1.50 (m, 2H), 1.31 – 1.13 (m, 8H), 1.05 (t, 6H, *J* = 6.6). HRMS *m/z* calcd for $C_{94}H_{94}F_4N_{16}O_{12} + 2H^+$: 858.36594, obsd 858.36643.

Dimeric ligand IVA. Yield after RP-HPLC purification: 2.06 mg (1.3 μmol, 9%). LC-MS analysis: t_R 7.94 min (gradient 30 to 90% B) and t_R 5.08 min (gradient 50 to 90% B). ESI-MS *m/z*: 1608.0 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.79 (s, 2H), 8.52 (s, 2H), 8.32 (br t, 2H), 7.91 (br t, 2H), 7.61 (d, 8H, *J* = 8.4), 7.48 – 7.32 (m, 12H), 7.22 – 7.05 (m, 12H), 6.14 (br t, 2H), 5.54 (s, 4H), 5.32 – 4.23 (m, 6H), 4.18 – 4.05 (m, 8H), 3.63 – 3.58 (m, 8H), 3.18 – 3.06 (m, 8H), 1.30 (t, 6H, *J* = 7.2), 1.05 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{86}H_{82}F_4N_{16}O_{12} + 2H^+$: 804.31899, obsd 804.31867.

Dimeric ligand IVB. Yield after RP-HPLC purification: 2.01 mg (1.2 μmol, 8%). LC-MS analysis: t_R 8.65 min (gradient 30 to 90% B) and t_R 5.82 min (gradient 50 to 90% B). ESI-MS *m/z*: 1637.2 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.79 (s, 2H), 8.52 (s, 2H), 8.31 (t, 2H, *J* = 3.6), 7.60 (d, 4H, *J* = 9.0), 7.50 – 7.38 (m, 12H), 7.20 – 7.05 (m, 12H), 6.13 (t, 2H*J* = 4.2), 5.53 (s, 4H), 5.31 (t, 2H, *J* = 3.6), 4.37 – 4.24 (m, 2H), 4.18 – 4.06 (m, 8H), 4.08 (d, 4H), 3.69 – 3.55 (m, 4H), 3.17 – 3.06 (m, 8H), 1.32 – 1.27 (m, 12H), 1.05 (t, 6H, *J* = 7.1). HRMS *m/z* calcd for $C_{88}H_{86}F_4N_{16}O_{12} + 2H^+$: 818.33464, obsd 818.33469.

Dimeric ligand IVC. Yield after RP-HPLC purification: 1.31 mg (0.8 μmol, 5%). LC-MS analysis: t_R 9.77 min (gradient 30 to 90% B) and t_R 7.36 min (gradient 50 to 90% B). ESI-MS *m/z*: 1693.3 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.80 (s, 2H), 8.52 (s, 2H), 8.48 – 8.42 (m, 2H), 7.72 (d, 2H, *J* = 9.0), 7.60 (d, 4H, *J* = 9.0), 7.48 – 7.02 (m, 24H), 6.11 (t, 2H, *J* = 4.2), 5.54 (s, 4H), 5.31 (t, 2H, *J* = 3.6), 4.38 (br d, 2H), 4.30 – 4.06 (m, 12H), 3.60 (dd, 4H, *J* = 63.6 , *J* = 19.8), 3.22 – 3.05 (m, 8H), 1.85 – 1.78 (m, 2H), 1.29 (t, 6H, *J* = 6.6), 1.04 (t, 6H, *J* = 7.2), 0.71 (d, 6H, *J* = 6.6), 0.63 (d, 6H, *J* = 6.6). HRMS *m/z* calcd for $C_{92}H_{94}F_4N_{16}O_{12} + 2H^+$: 846.36594, obsd 846.36576.

Dimeric ligand IVD. Yield after RP-HPLC purification: 2.44 mg (1.5 μmol, 10%). LC-MS analysis: t_R 7.95 min (gradient 30 to 90% B) and t_R 4.92 min (gradient 50 to 90% B). ESI-MS *m/z*: 1665.1 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.79 (s, 2H), 8.54 (s, 2H), 8.26 (t, 2H, *J* = 5.4), 7.64 (t, 2H, *J* = 5.4), 7.60 (d, 4H, *J* = 9.0), 7.49 – 7.39 (m, 14H), 7.20 – 6.97 (m, 10H), 6.17 (t, 2H, *J* = 5.4), 5.53 (s, 4H), 5.31 (br t, 2H), 4.32 – 4.25 (m, 8H), 4.15 – 4.05 (m, 6H), 3.53 (s, 4H), 3.13 – 3.02 (m, 8H), 2.90 – 2.86 (m. 4H), 2.03 – 1.98 (m, 4H), 1.56 – 1.49 (m, 4H), 1.30 (t, 6H, *J* = 7.2), 1.04 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{90}H_{90}F_4N_{16}O_{12} + 2H^+$: 832.35029, obsd 832.35036.

Dimeric ligand IVE. Yield after RP-HPLC purification: 2.04 mg (1.2 μmol, 8%). LC-MS analysis: t_R 9.25 min (gradient 30 to 90% B) and t_R 6.76 min (gradient 50 to 90% B). ESI-MS *m/z*: 1720.1 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.80 (s, 2H), 8.54 (s, 2H), 8.27 (br t, 4H), 7.58 (d, 4H, *J* = 9.0), 7.55 – 7.28 (m, 12H), 7.21 – 7.05 (m, 12H), 6.15 (t, 2H), 5.54 (s, 4H), 5.33 (s, 4H), 4.28 (q, 4H, *J* = 6.6), 4.08 (d, 4H, *J* = 4.8), 3.56 – 3.52 (m, 8H), 3.14 – 3.09 (m, 4H), 3.00 (s, 4H), 2.71 – 2.65 (m, 4H), 2.04 (t, 4H, *J* = 9.0), 1.43 – 1.38 (m, 4H), 1.31 (t, 6H, *J* = 7.2), 1.26 – 1.15 (m, 8H), 1.05 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{94}H_{98}F_4N_{16}O_{12} + 2H^+$: 871.37256, obsd 871.37218.

Dimeric ligand IVF. Yield after RP-HPLC purification: 2.60 mg (1.5 μmol, 10%). LC-MS analysis: t_R 9.14 min (gradient 30 to 90% B) and t_R 6.39 min (gradient 50 to 90% B). ESI-MS *m/z*: 1688.9 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.81 (s, 2H), 8.51 (s, 2H), 8.27 (br s, 2H), 7.60 (d, 4H, *J* = 9.0), 7.49 – 7.08 (m, 20H), 6.16 (br s, 2H), 5.56 (s, 4H), 5.31 (t, 2H), 4.39 – 4.32 (m, 2H), 4.30 – 4.23 (m, 4H), 4.18 – 4.00 (m, 5H), 3.70 – 3.58 (m, 4H), 3.56 (s, 4H), 3.15 – 3.05 (m, 4H), 2.05 – 1.96 (m, 2H), 1.95 – 1.80 (m, 2H), 1.72 – 1.68 (m, 2H), 1.66 – 1.50 (m, 2H), 1.29 (t, 6H, *J* = 7.2), 1.04 (t, 6H, *J* = 7.2). HRMS *m/z* calcd for $C_{92}H_{90}F_4N_{16}O_{12} + 2H^+$: 844.35029, obsd 844.35040.

Dimeric ligand IVG. Yield after RP-HPLC purification: 2.04 mg (1.2 μmol, 8%). LC-MS analysis: t_R 9.09 min (gradient 30 to 90% B) and t_R 6.61 min (gradient 50 to 90% B). ESI-MS *m/z*: 1716.2 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.82 (s, 2H), 8.50 (s, 2H), 8.26 (s, 2H), 7.59 (d, 4H, *J* = 7.8), 7.57 – 7.32 (m, 12H), 7.31 – 7.06 (m, 12H) 6.15 (s, 2H), 5.55 (s, 4H), 5.31 (t, 2H), 4.51 – 4.23 (m, 8H), 4.18 – 4.09 (m, 6H), 3.62 – 3.58 (m, 6H), 3.28 – 3.20 (m, 2H), 3.18 – 3.08 (m, 8H), 2.60 – 2.52 (m, 2H), 2.30 – 2.12 (m, 4H), 1.65 – 1.50 (m, 4H), 1.49 – 1.39 (m, 2H), 1.38 – 1.23 (m, 8H), 1.05 (t, 6H, *J* = 7.1). HRMS *m/z* calcd for $C_{94}H_{94}F_4N_{16}O_{12} + 2H^+$: 858.36594,

obsd 858.36579.

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Chapter 4

GnRHR binding and antagonism of dimeric systems appear dependent on the nature of the parent pharmacophore

Introduction

The gonadotropin-releasing hormone receptor (GnRHR) belongs to the family of G protein coupled receptors (GPCR) and is expressed in the anterior pituitary gland. Upon stimulation with the decapeptidic agonist GnRH, the gonadotropins lutheinizing hormone (LH) and follicle-stimulating hormone (FSH) are secreted. LH controls ovulation in women and testosterone production in men. FSH is involved in spermatogenesis and induces ovarian follicle growth. Abnormalities in signaling of GnRHR may result in the malfunction of steroid production and ovarian regulation.[1,2] Literature evidence suggests that GnRHR signaling is associated with receptor dimerization.[3-7] Chapters 2 and 3 describes the design, synthesis and evaluation of dimeric ligands derived from the known GnRHR antagonist imidazopyrimidinone **1** (Figure 1).[8,9] Biological evaluation revealed that in the functional assay, a significant drop in antagonistic potencies of the functionalized ligands was observed. Interestingly, the binding affinities of the dimeric ligands were in the same order of magnitude or only slightly impaired when compared to the monomeric reference compounds.

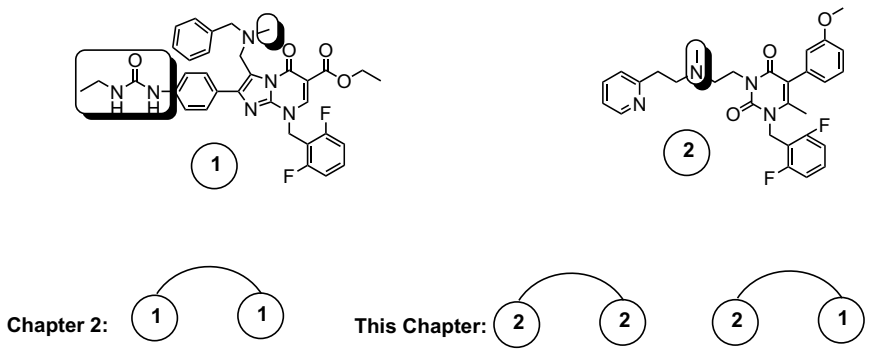


Figure 1. Schematic representation of the dimeric ligands from Chapter 2 and this Chapter.

The discrepancy between binding affinity and antagonism for these dimeric ligands may be caused by several reasons. For instance, the nature of the linker by which the two monomers are interconnected may have an influence. In this respect, two sets of linkers based on either oligoethylene glycol[8] or a bis-substituted benzene as the core[9] of the linker system were prepared. Obviously, the whole concept of dimeric GnRHR antagonists may be at odds with the intrinsic GnRHR mediated signaling pathway. However, the current knowledge on the biological pathway does not warrant this conclusion at this stage,[3-7] and moreover, there is ample chemical space yet open for the development of alternative potential dimeric ligands.[10,11] In fact it may well be that the previously reported decrease of antagonism with dimers of imidazopyrimidinone **1** is due to intrinsic properties of the parent ligand, and not caused by the nature of their interconnection. With this thought in mind, a second GnRHR antagonist was included with a structurally distinct chemotype in the dimeric ligand studies. For reasons of both synthetic availability and the amenability to further functionalization GnRHR antagonist **2** was selected.[12,13] This Chapter presents the results in the preparation of homodimeric ligands containing two copies of **2**, as well as so-called 'heterodimeric' compounds containing both **1** and **2** (Figure 2). The propensity of these to both bind and antagonize the GnRHR is evaluated and the results are compared to those described previously on homo-dimeric ligands containing solely pharmacophore **1** (see for a generalized impression on the respective homo- and hetero-dimers Figure 1).

Results and Discussion

The construction of dimeric ligands (**5a-e** and **6a-e**) by copper (I) catalyzed 1,3-dipolar Huisgen cycloaddition of acetylene functionalized ligands **3** or **4** with a set of flexible oligoethylene derived bis-azides was described in Chapter 2.[8] The facile preparation of the various $PEG(N_3)_2$ molecules and the ease with which either one or two azides (controlled by stoichiometry) partake in an ensuing Huisgen [2 + 3]-cycloaddition event made us decide to implement these linker molecules also in the generation of homo- and heterodimers bearing pharmacophore **2**. Specifically, constructs **9a-e** (the homodimers based on **2**), **8a-e** (monomeric **2** linked to the spacers for control experiment purposes), **10a-e** and **11a-e** (heterodimers containing antagonist **2** linked to **1** at two different positions) were synthesized.

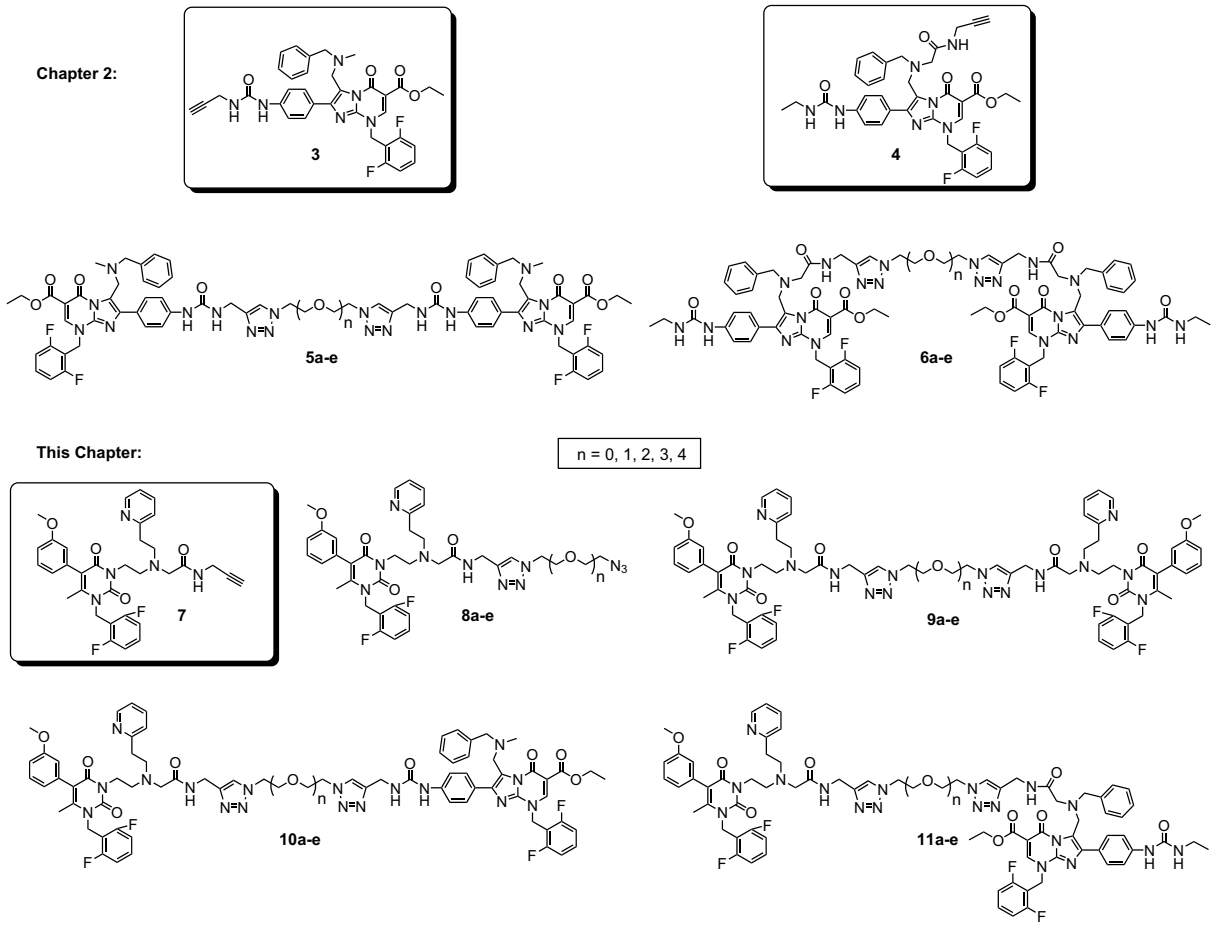
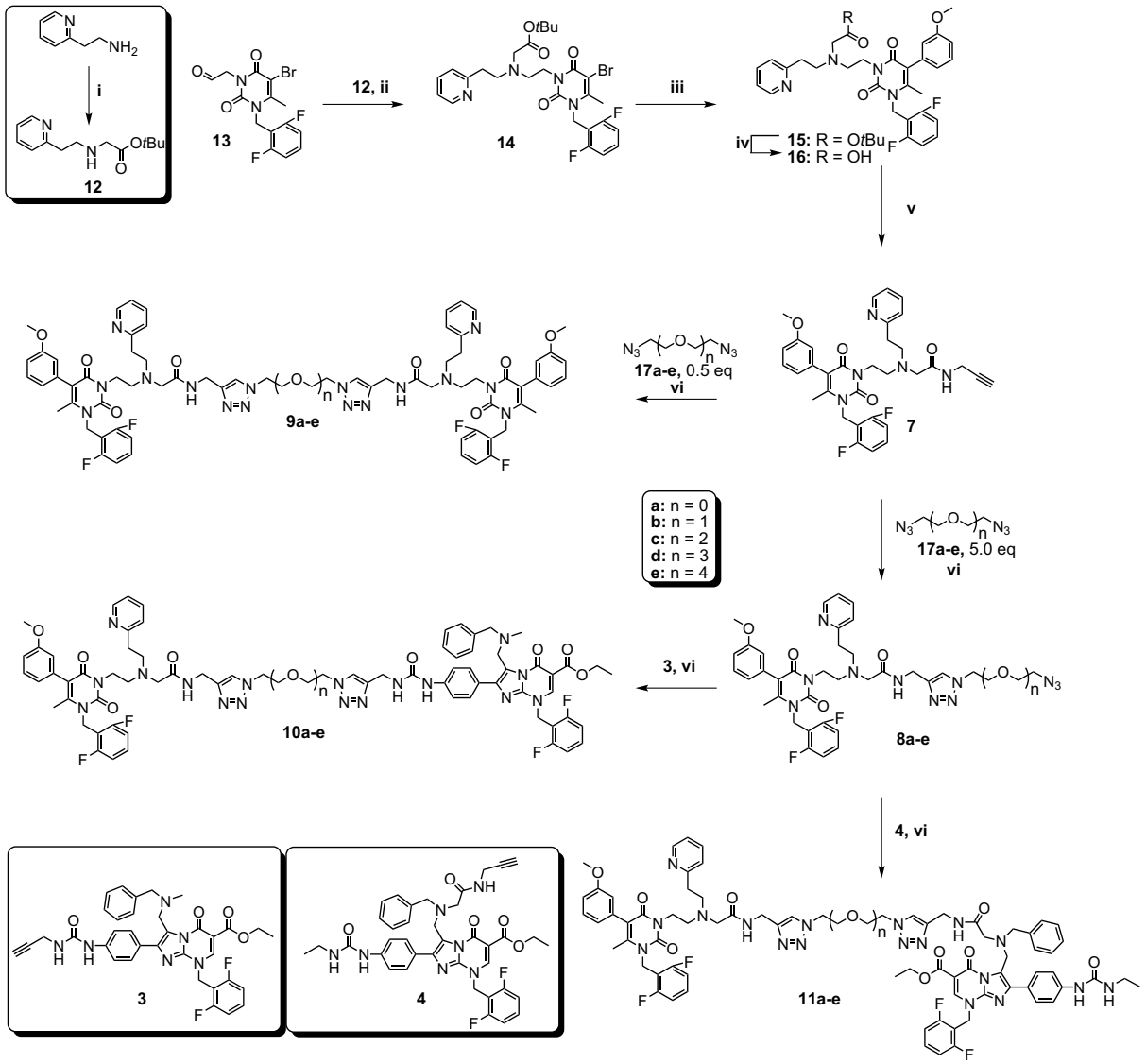


Figure 2. Structures of all compounds described in this Chapter.

These compounds were pharmacologically evaluated and compared with two members from the previously prepared libraries, **5c** and **6c**, encompassing homodimeric compounds assembled from **1**.[8] The synthesis of acetylene functionalized uracil derivative **7**, required for the construction of the compound library, was accomplished by adapting a known procedure to antagonist **2** (see Scheme 1).[12,13] Thus, reductive amination of aldehyde **13** (prepared as described in reference 12) with *tert*-butyl (pyridinylethylamino)acetate **12** gave bromide **14** in a 88% yield. Subsequent Suzuki coupling of **14** with 3-methoxy phenyl boronic acid in a toluene/acetonitrile/water mixture and K_2CO_3 at elevated temperature afforded **15** in 70% yield. Acidic removal of the *tert*-butyl ester in **15** (TFA/DCM) followed by standard peptide coupling of **16** with propargyl amine, afforded acetylene **7** in 95% over the two steps.

The series of dimeric ligands **9** were prepared using two equivalents of acetylene **7** to bis-azide spacer (**17a-e**) in the presence of 0.2 equivalents of copper sulphate and one equivalent of sodium ascorbate at elevated temperature. Alternatively, the use of a five-fold excess of diazide spacers (**17a-e**) led to the formation of mainly the monomeric products **8a-e**. The heterodimeric ligands **10a-e** and **11a-e** were obtained by Huisgen [2+3]-cycloaddition of azides **8a-e** with acetylene functionalized **3** and **4**,[8,14-16] respectively.



Scheme 1. Synthesis of homo- and heterodimeric GnRHR ligands. *Reagents and conditions*: *i. tert*-butyl bromoacetate, DiPEA, THF, 76%; *ii.* $NaBH(OAc)_3$, DCE, 16h, 88%; *iii.* 3-methoxy phenylboronic acid, K_2CO_3, $Pd(PPh_3)_4$, toluene/CH_3CN/H_2O; 2/2/1; v/v/v, 90 °C, on, 70%; *iv.* TFA/DCM ; 1/2 ; v/v, on, quant; *v.* propargylamine, BOP, DiPEA, DMF, on, 95%; *vi.* 0.2 eq $CuSO_4$, 1 eq sodium ascorbate, *t*BuOH/CH_3CN/H_2O; 2/2/1; v/v/v, 60 °C, 2h.

All synthesized compounds were tested on their ability to bind to the GnRH receptor using the in Chapter 2 described binding assay.[8] As can be seen from Table 1, modification of the tertiary amine in **2** has a detrimental effect on the GnRHR binding affinities. Acetylene **7** has a 20-fold decrease in affinity for the GnRHR when compared to **2** and this loss in binding affinity is even more pronounced in the oligo-ethylene glycol-modified derivatives **8a-e**. Introducing a second copy of the parent ligand, as in **9a-d**, restores binding affinity to some extent. This is not observed for ligand **9e**, possessing the longest spacer length. The two series of hetero-dimeric ligands **10a-e** and **11a-e** are comparatively stronger GnRHR binders and it may well be that the higher GnRHR affinity of **1** compared to **2** is at the basis of this increase in binding affinity. Indeed, the highest binding affinities in these series approach those found for **5c** and **6c**, the most potent

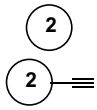
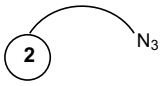
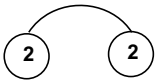
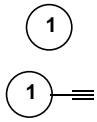
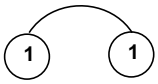
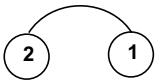
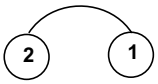
Compound		n	K_i (nM)[a]	IC_{50} (nM)[b]
	*Monomeric ligands from **2***			
	**2**	-	31	476
	7	-	616	3160
	8a	0	2107	>10000 (44 ± 4)
	8b	1	1541	>10000 (50 ± 6)
	**8c**	2	1403	>10000 (39 ± 3)
	8d	3	2254	>10000 (38 ± 2)
	8e	4	1323	>10000 (37 ± 0)
	*Homodimeric ligands with **7***			
	9a	0	990	746
	9b	1	721	576
	**9c**	2	962	783
	9d	3	966	766
	9e	4	1651	857
	*Monomeric ligands from **1***			
	**1**	-	4.8	76
	3	-	1.4	724
	4	-	5.2	1134
	*Homodimeric ligands with **3** or **4***			
	**5c**	2	24	755
	6c	2	61	849
	*Heterodimeric ligands with **7** and **3***			
	10a	0	133	708
	10b	1	129	426
	**10c**	2	121	769
	10d	3	43	631
	10e	4	26	1460
	*Heterodimeric ligands with **7** and **4***			
	11a	0	117	320
	11b	1	45	278
	**11c**	2	128	396
	11d	3	187	447
	11e	4	67	664

Table 1. Binding affinities and functional antagonistic potencies of all compounds. n = number of ethylene glycol units in the spacer. [a]Membranes of GnRHR-expressing CHO cells were incubated with the radioactive GnRHR agonist [^{125}I]triptorelin. K_i values were determined by incubating the membranes with increasing concentrations of the test compound and calculated based on the concentration of the compound needed to displace 50% of radioligand [^{125}I]triptorelin. The mean K_i are calculated from the -log K_i values from two or three independent experiments performed in duplicate. The SD of pK_i is generally lower than 0.2.; [b]CHO cells that stably express the GnRHR were stimulated with a submaximal (EC_{80}) concentration of GnRH and were incubated with increasing concentrations of the compounds. The IC_{50} value is the concentration of compound needed to inhibit the agonistic response by 50%. The mean IC_{50} are calculated from the -log IC_{50} values from two or three independent experiments performed in duplicate. The SD of pIC_{50} is generally lower than 0.2.; For compounds **8a-e**, the percentage of inhibition at a concentration of 10 μM of the test compound are represented in brackets.

homodimers reported previously, composed of two copies of **3** and **4,** respectively. It may be concluded that a loss in binding affinity caused by functionalization of a monomer (for instance, **2** to **8**) can indeed be restored by introduction of a second pharmacophoric element (for instance, **8** to **9**).

The antagonistic potencies of the compound library were measured in a functional assay that was also described in Chapter 2.[8] The highest concentration of test compound measured in this assay is 10 μM and for compounds **8a-e**, less than 50% antagonistic efficacy was observed at this concentration.[17] This indicates that the IC_{50} values for the monomeric compounds **8a-e** are at least five times higher than the corresponding K_i values (Table 1). Homodimers **9a-e** appears at least one order of magnitude more potent than their monomeric counterparts **8a-e** in this assay. Notably, the antagonistic potency of the dimeric ligands **9a-e** is in the same range as the binding affinities for the dimeric ligands. This phenomenon seems independent of the spacer length between the two ligands.
The potencies of the hetero-dimeric ligands **10a-e** are similar to those of the homodimeric ligands **9a-e**. The attachment-site of antagonist **1** seems significant and a two-fold increase in potency is observed for compounds **11a-e**. Introducing bivalency might affect the intrinsic activity of the compounds and thus change their profile from an antagonist into an agonist.[7] Additional assays performed with all compounds in an agonistic set-up did not provide any actives (data not shown).

The present study reveals that the presence of a second pharmacophore in dimeric ligands based on **2** strongly influences the pharmacological properties. For example, dimeric ligands **9a-e** are at least ten-fold more potent GnRHR antagonists compared to **8a-e**. Remarkably, the binding affinity for most dimeric ligands (that is, **9a-d**) increased only two-fold compared to **8a-d**. The exception of this trend is found in dimeric ligand **9e**, which does not show an increase in binding affinity compared to **8e**. This result can be explained by the distance between the two pharmacophores, which is largest in **9e**. Possibly, binding of a second ligand in **9e** to the receptor becomes less favorable for entropy reasons with increasing spacer-length.[18]
The affinity and antagonistic potency of the prepared compounds are not enhanced compared to the rigid, entropically favorable parent structure **2**. Introduction of an acetylene function at the methyl amine position (**2** to **7**) already gives a decrease in affinity and antagonistic effect and this loss in activity becomes even more prominent when the spacer function is introduced (**8a-e**). However, the fact that both the affinity and antagonistic potency of the compounds increased upon introduction of a second ligand is reason to conclude that a bivalency effect is present in this series.
The heterodimeric molecules **10a-e** and **11a-e**, incorporating a copy of both pharmacophores **1** and **2**, are more potent GnRHR binders and antagonists than homodimers **9a-e**. In Chapter 2, it was described that dimeric ligands based on two copies of **1** have high binding affinities, while the antagonistic potencies are not that high. It can therefore be hypothesized that the recognition unit

derived from **1** is at least partly responsible for binding of the hetero-dimeric ligands to the receptor.

Recent literature evidenced that TAK013,[19] a GnRHR antagonist that is based on a thienopyrimidinone scaffold, is an insurmountable antagonist for the human GnRHR.[20] Since the scaffold and peripheral decoration of TAK013 highly resemble that of the imidazopyrimidinone **1**, it is not excluded that ligands containing **1** behave in a similar fashion.[21-24] When it is true that heterodimeric ligands possessing **1** as recognition unit have one slowly dissociating ligand, this may explain the high GnRHR binding affinities observed for the compounds, that appear at odds with the antagonistic potencies.

Conclusion

This Chapter describes the synthesis and evaluation of homo-and heterodimeric ligands for the GnRHR. The most potent antagonists described here are dimeric ligands that are interconnected with one ethyleneglycol spacer unit (n=1). This holds true for both homo-dimers and hetero-dimers. This spacer may thus possess the optimal distance for interaction of two ligands to a GnRHR (or a dimer). The question remains whether the dimeric ligands bind to two distinct receptors or if the increased pharmacological properties originate from an increased local concentration that is attained upon monovalent binding of the dimeric ligands.[8] Since none of the dimeric ligands described in this Chapter has a significantly improved pharmacological profile or altered intrinsic activity (that is, from antagonist to agonist)[7] compared to ligands **2** or **1**, no definite conclusions on the binding mode can be made at this stage. Further exploration of the chemical space with respect to alternative ligand/GPCR complexes and/or spacer systems may reveal more conclusive information on this subject.

Experimental procedures

GnRHR Luciferase reporter gene assay

Chinese Hamster Ovary, CHO-K1, cells with stable expression of the human Gonadotropin-Releasing Hormone Receptor (GnRHR) and Nuclear Factor Activated T-cell luciferase reporter gene were grown to 80-90% confluence in culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% w/v Fetal Bovine Serum, 100 units/mL Penicillin and 100 µg/mL Streptomycin and 400 µg/mL Geniticin. On the day of the assay, cells were washed twice with Phosphate Buffered Saline and then harvested with cell dissociation solution. Cells were resuspended in assay medium consisting of DMEM supplemented with 1 mg/L insulin and 5mg/L apo-transferrin and 3% v/v DMSO. Then, 10 µL cell suspension containing 7,500 cells was added to each well of a 384-well white culture plate. Thereafter, 10 µL of test compound was added at 10 concentrations ranging from final concentration of 10 µM to 0.3 nM with half log intervals. Compounds were allowed to preincubate with cells for 30 min followed by addition of 10 µL agonist GnRH at a final concentration of 3 nM which produces approximately 80% of the maximal effect (EC_{80}) when given alone. After 4 h stimulation, 15 µL of luclite® was added to each well

for detection of luciferase protein and plates were left at room temperature for 1 h in the dark. Finally, the luminescence signal was quantified on the TopCount® Microplate Scintillation and Luminescence Counter.

Radioligand Binding Assay.

Ganirelix was provided by Schering-Plough research institute (Oss, The Netherlands). [^{125}I]triptorelin (specific activity 2200 Ci mmol^{-1}) was purchased from Perkin Elmer Life Sciences B.V. (Groningen, The Netherlands). CHO-K1 cells stably expressing the human GnRH receptor were provided by Schering-Plough research institute (Oss, The Netherlands). All other chemicals and cell culture materials were obtained from standard commercial sources.

CHO (Chinese hamster ovary) -K1 cells expressing the wild-type human GnRH receptor were grown in Ham's F12 medium containing 10% bovine calf serum, streptomycin (100 µg mL^{-1}), penicillin (100 IU mL^{-1}) and G418 (0.4 mg mL^{-1}) at 37 °C in 5% CO_2. The cells were subcultured twice weekly at a ratio of 1:20. For membrane preparation the cells were subcultured 1:10 and transferred to large 14-cm diameter plates. For membrane preparation the cells were detached from the plates by scraping them into 5 mL PBS, collected and centrifuged at 1400 *g* (3000 rpm) for 5 min. Pellets derived from 30 plates were pooled and resuspended in 30 mL of ice-cold 50 mM Tris-HCl buffer supplemented with 2 mM $MgCl_2$, pH 7.4. An UltraThurrax was used to homogenize the cell suspension. Membranes and the cytosolic fraction were separated by centrifugation at 100,000 *g* (31,000 rpm) at 4 °C for 20 min. The pellet was resuspended in 10 mL of the Tris-HCl buffer and the homogenization and centrifugation steps were repeated. Tris-HCl buffer (10 mL) was used to resuspend the pellet and the membranes were stored in 500 µL aliquots at -80 °C. Membrane protein concentrations were measured using the BCA (bicinchoninic acid) method.[25]

On the day of the assay membrane aliquots containing 20 µg protein were incubated in a total volume of 100 µL assay buffer (50 mM Tris HCl, pH 7.4, supplemented with 2 mM $MgCl_2$ and 0.1% BSA) at 22 °C for 45 min. Displacement experiments were performed using five concentrations of competing ligand in the presence of 30,000 cpm [^{125}I]triptorelin. Non-specific binding was determined in the presence of 1 µM ganirelix and represented approximately 20% of the total binding. Total binding was determined in the presence of buffer and was set at 100% in all experiments, whereas non-specific binding was set at 0%. Incubations were terminated by dilution with ice-cold Tris HCl buffer. Separation of bound from free radioligand was performed by rapid filtration through Whatman GF/B filters pre-soaked with 0.25 % PEI for 1 h using a Brandel harvester. Filters were subsequently washed three times with ice-cold wash buffer (50 mM Tris HCl, pH 7.4, supplemented with 2 mM $MgCl_2$ and 0.05% BSA). Filter-bound radioactivity was determined in a γ-counter.

All data was analyzed using the non-linear regression curve-fitting program GraphPad Prism v. 4 (GraphPad Software Inc, San Diego, CA, U.S.A.). Inhibitory binding constants (K_i values) were derived from the IC_{50} values according to $K_i = IC_{50}/(1 + [C]/K_d)$ where [C] is the concentration of the radioligand and K_d its dissociation constant.[26] The K_d value (0.35 nM) of [^{125}I]triptorelin was obtained by computer analysis of saturation curves.[27] All values obtained are means of at least two independent experiments performed in duplicate.

Cytotoxicity. CHOhGnRH_luc cells were seeded on 5-cm diameter plates in assay medium in the absence (control) or presence of 10 µM of test compounds. Compounds **8c**, **9c**, **10c** and **11c** were selected as relevant compounds in this toxicity assay. The cells were incubated for 4 h at 37 °C. Thereafter the cells were harvested using 0.5 mL trypsol and resuspended in 2 mL of PBS. Subsequently the number of viable cells was determined by trypan blue exclusion, where a trypan blue solution (0.8 % (w/v) in PBS) was added to an equal amount of cell suspension. The proportion of live cells was determined by counting in a hemocytometer.

Chemical procedures.

Reactions were executed at ambient temperatures unless stated otherwise. All moisture sensitive reactions were performed under an argon atmosphere. All solvents were removed by evaporation under reduced pressure. Reactions were monitored by TLC analysis using silica gel coated plates (0.2 mm thickness) and detection by 254 nm UV-light or by either spraying with a solution of $(NH_4)_6Mo_7O_{24} \times 4H_2O$ (25 g/L) or $(NH_4)_4Ce(SO_4)_4 \times 2H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at ~150 °C. Column chromatography was performed on silica gel (40-63 μm). NMR spectra were recorded on a 200/50 MHz, 300/75 MHz, 400/100 MHz, 500/125 MHz or 600/150 MHz spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants (*J*) are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. For LC-MS analysis, a HPLC-system (detection simultaneously at 214 and 254 nm) equipped with an analytical C_{18} column (4.6 mmD × 250 mmL, 5μ particle size) in combination with buffers A: H_2O, B: CH_3CN and C: 1% aq. TFA and coupled to a mass instrument with an electronspray interface (ESI) was used. For RP-HPLC purifications, an automated HPLC system equipped with a preperative C_{18} column (5 μm C_{18}, 10Å, 150 × 21.2 mm) was used. The applied buffers were A: H_2O + 0.1% TFA and B: CH_3CN. High resolution mass spectra were recorded by direct injection (2 μL of a 2 μM solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60000 at *m/z* 400 (mass range *m/z* = 150-2000) and dioctylpthalate (*m/z* = 391.28428) as a lock mass. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan).

***tert*-Butyl 2-(2-(pyridin-2-yl)ethylamino)acetate (12)**. To a cooled (0 °C) solution of 2-(2-pyridyl)ethylamine (4.79 mL, 40 mmol) and *tert*-butyl bromoacetate (2.95 mL, 20 mmol) in THF (20 mL) was added DiPEA (1.70 mL, 10 mmol). The mixture was stirred overnight at rt. The slurry was evaporated and dissolved in EtOAc (100 mL). The organic layer was washed with aqueous saturated $NaHCO_3$ solution (50 mL) dried on $MgSO_4$ and evaporated. The crude liquid was purified by silica gel column chromatography (0 to 20% MeOH in EtOAc) to yield **12** in 76% yield (3.6 g, 15.3 mmol). ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.00 (d, 1H, *J* = 4.4, CH Ar), 7.05 (t, 1H, *J* = 7.3, CH Ar), 6.68 – 6.54 (m, 2H, 2 × CH Ar), 2.81 (s, 2H, CH_2), 2.51 – 2.46 (m, 4H, 2 × CH_2), 1.80 (br s, 1H, NH), 0.92 (s, 9H, *t*Bu). ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.3, 159.0 (2 × C), 148.2, 135.2, 122.1, 120.1 (4 × CH), 79.2 (C), 50.6, 47.8, 37.4 (3 × CH_2), 26.9 (CH_3). ESI-MS *m/z*: 236.93 $[M + H]^+$.

***tert*-Butyl 2-((2-(5-bromo-3-(2,6-difluorobenzyl)-4-methyl-2,6-dioxo-2,3-dihydropyrimidin-1(6H)-yl)ethyl)(2-(pyridin-2-yl)ethyl)amino)acetate (14)**. To a solution of aldehyde **13** (2.46 g, 6.7 mmol) and amine **12** (1.77 g, 7.5 mmol) in dichloroethane (50 mL) was added $NaBH(OAc)_3$ (2.84 g, 13.4 mmol) in portions. The reaction was stirred overnight and diluted with CH_2Cl_2 (50 mL). The organic layer was washed with water (50 mL), brine (50 mL), dried ($MgSO_4$) and concentrated. The crude solid was purified by silica gel column chromatography (33 to 66% EtOAc in toluene) to yield bromide **14** in 88% yield (3.5 g, 5.9 mmol). ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.00 (d, 1H, *J* = 4.4, CH Ar), 7.05 (t, 1H, *J* = 7.3, CH Ar), 7.28 – 7.03 (m, 3H, 3 × CH Ar), 6.68 (t, 2H, *J* = 8.8, 2 × CH Ar), 5.26 (s, 2H, CH_2), 4.07 (t, 2H, *J* = 6.6, CH_2), 3.37 (s, 2H, CH_2), 3.11 – 2.82 (m, 6H, 3 × CH_2), 2.49 (s, 3H, CH_3), 1.44 (s, 9H, *t*Bu). ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.4, 162.9, 159.8, 158.0, 150.3, 149.3 (6 × C), 148.5, 135.7 (2 × CH), 129.6 (t, *J* = 10.6, CH), 122.9, 120.5 (2 × CH), 111.3 (d, 2H, *J* = 24.3, 2 × CH), 110.7, 98.7, 80.2 (3 × C), 55.1, 53.6, 50.1, 40.3, 39.2, 36.5 (6 × CH_2), 27.7 (3 × CH_3), 19.7 (CH_3). ESI-MS *m/z*: 593.07, 595.07 $[M + H]^+$.

***tert*-Butyl 2-((2-(3-(2,6-difluorobenzyl)-5-(3-methoxyphenyl)-4-methyl-2,6-dioxo-2,3-dihydropyrimidin-1(6H)-yl)ethyl)(2-(pyridin-2-yl)ethyl)amino)acetate (15)**. A solution of bromide **14** (2.36 g, 4.0 mmol), 3-methoxyphenylboronic acid (0.91 g, 6.0 mmol), K_2CO_3 (1.24 g, 9.0 mmol) in 25 mL toluene/acetonitrile/water (2/2/1; v/v/v) was degassed for 1 h. $Pd(PPh_3)_4$ (0.46 g, 0.4 mmol) was added and the mixture was heated overnight at 90 °C. EtOAc (100 mL) and water were added (50 mL) and the organic layer was washed with water (2 × 50 mL), brine (50 mL), dried ($MgSO_4$) and evaporated. The crude solid was purified by silica gel column chromatography (10 to 25% EtOAc in toluene) to yield **15** in 70% yield (1.8 g, 2.8 mmol) as an off-white solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.43 (d, 1H, *J* = 4.4, CH Ar), 7.67 – 7.35 (m, 3H, CH Ar), 7.28 – 6.97 (m, 4H, CH Ar), 6.82 – 6.69 (m, 3H, CH Ar), 5.16 (s, 2H, CH_2), 4.04 (t, 2H, *J* = 6.6, CH_2), 3.73 (s, 3H, OMe), 3.35 (s, 2H, CH_2), 3.06 – 2.81 (m, 6H, 3 × CH_2), 2.10 (s, 3H, CH_3), 1.38 (s, 9H, *t*Bu). ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.4 (C), 163.1 (d, 1C, *J* = 9.1), 161.5, 159.9, 159.2 (3 × C), 158.2 (d, 1C, *J* = 7.6), 151.1 (C), 148.5 (CH), 148.1 (C), 135.9 (CH Ar), 135.2 (C), 131.7, 129.0, 128.3, 123.0, 120.7, 116.1 (6 × CH Ar), 114.1 (C), 111.5 (d, *J* = 24.3, 2 × CH Ar), 80.2 (C), 55.3 (CH_2), 54.7 (CH_3), 53.9, 50.4, 39.2, 38.4, 36.5 (5 × CH_2), 27.7 (3 × CH_3), 17.2 (CH_3). ESI-MS *m/z*: 621.13 $[M + H]^+$.

2-((2-(3-(2,6-Difluorobenzyl)-5-(3-methoxyphenyl)-4-methyl-2,6-dioxo-2,3-dihydropyrimidin-1(6H)-yl)ethyl)(2-(pyridin-2-yl)ethyl)amino)-N-(prop-2-ynyl)acetamide (7). Compound **15** (1.0 g, 1.6 mmol) was dissolved in a TFA/DCM mixture (40 mL, 1/2) and stirred overnight at rt. Toluene was added (25 mL) and the mixture was evaporated and additionally coevaporated with toluene (2 × 25 mL). The crude product (**16**) was dissolved in DMF (20 mL) and propargyl amine (0.13 g, 2.4 mmol), BOP (1.4 g, 3.2 mmol) and DiPEA (1.5 mL, 8.0 mmol) were subsequently added. The reaction mixture was stirred overnight and the mixture was evaporated. EtOAc (50 mL) and aqueous saturated $NaHCO_3$ solution (25 mL) was added and the organic layer was washed with aqueous saturated $NaHCO_3$ solution (2 × 25 mL), water (25 mL), brine (25 mL), dried on $MgSO_4$ and evaporated. The crude solid was purified by silica gel column chromatography (50 to 100% EtOAc in toluene) to yield **7** in 95% yield (0.91 g, 1.5 mmol) as an off-white solid. LC-MS analysis: t_R 6.35 min (linear gradient 10 to 90% B in 14.5 min; *m/z*: 602.27 $[M + H]^+$). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.41 (d, 1H, *J* = 5.0, CH Ar), 7.64 (t, 1H, *J* = 5.5, CH Ar), 7.55 (dd, 1H, *J* = 12.0, *J* = 7.5, CH Ar), 7.20 – 7.09 (m, 2H, CH Ar), 7.05 (d, 1H, *J* = 7.5, CH Ar), 7.00 (dd, 1H, *J* = 7.0, *J* = 5.0, CH Ar), 6.81 – 6.74 (m, 3H, CH Ar), 6.66 (d, 1H, *J* = 7.5, CH Ar), 6.63 (s, 1H, CH Ar), 5.16 (s, 2H, CH_2), 3.92 (t, 2H, *J* = 6.6, CH_2), 3.82 (dd, 2H, *J* = 5.5, *J* = 2.0, CH_2), 3.66 (s, 3H, OMe), 3.14 (s, 2H, CH_2), 2.82-2.78 (m, 4H, 2 × CH_2), 2.71 (t, 2H, *J* = 6.5, CH_2), 2.09 (t, 1H, *J* = 3.0, CH), 2.04 (s, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.8 (C), 161.6 (d, *J* = 9.1, 1 × C), 161.5 (C), 159.6 (d, *J* = 7.6, 1 × C), 159.4, 159.2, 151.2 (3 × C), 148.7 (CH), 148.4 (C), 136.1 (CH Ar), 134.9 (C), 131.6 (CH Ar), 129.4 (t, *J* = 10.4,, CH), 129.1, 123.1, 122.7, 116.2, (5 × CH Ar), 114.1 (C), 112.9 (CH Ar), 111.5 (d, *J* = 25.5, 2 × CH Ar), 79.7 (C), 70.3 (CH), 58.7 (CH_2), 54.6 (CH_3), 54.3, 51.2, 39.2, 38.5, 35.3, 28.1 (6 × CH_2), 17.3 (CH_3). HRMS *m/z* calcd for $C_{33}H_{33}N_5O_4F_2 + H^+$: 602.25734, obsd 602.25728.

***General method for the preparation of monomeric ligands 8a-e*.**

A solution of the acetylene functionalized ligand **7** (90.2 mg, 0.15 mmol) and bis-azide spacer **17a, 17b, 17c, 17d** or **17e** (0.75 mmol) in a mixture of *t*BuOH/CH_3CN/H_2O (2/2/1; v/v/v, 1 mL) was degassed for 1h. Sodium ascorbate (1 eq, 150 µL of a 1M solution in degassed H_2O) and $CuSO_4$ (0.2 eq, 150 µL of a 0.2M solution in degassed H_2O) were added and the reaction mixture was stirred at 60°C for 3 h. The mixtures were diluted with MeOH/$CHCl_3$ (25 mL, 1/9) and washed with water (10 mL). The waterlayer was extracted once with MeOH/$CHCl_3$ (25 mL, 1/9). The combined organic layers were dried ($MgSO_4$) and evaporated. The crude products were purified by silica gel column chromatography (0 to 20% MeOH in EtOAc). An analytically pure sample for biological evaluation was prepared by additional purification on preparative RP-HPLC system (linear gradient of 3.0 CV; 30

to 60% B).Evaporation and lyophilization of the combined fractions furnished monovalent ligands **8a-e** as white amorphous powders.

Monomeric ligand 8a. Yield after column purification: 62 mg (87 µmol, 58%). LC-MS analysis: t_R 6.33 min (linear gradient 10 to 90% B in 14.5 min; *m/z*: 714.33 $[M + H]^+$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.63 (d, *J* = 5.0, 1H, CH Ar), 8.21 (br s, 1H, NH), 8.08 (t, *J* = 7.6, 1H, CH Ar), 7.72 – 7.71 (m, 2H, CH Trl, CH Ar), 7.56 (t, *J* = 6.0, 1H, CH Ar), 7.35 – 7.23 (m, 2H, 2 × CH Ar), 6.93-6.88 (m, 3H, 3 × CH Ar), 6.76 – 6.73 (m, 2H, 2 × CH Ar), 5.24 (s, 2H, CH_2), 4.51 (d, 2H, *J* = 4.4, CH_2), 4.38 (t, 2H, *J* = 5.6, CH_2), 4.22 (t, 2H, *J* = 5.6, CH_2), 3.81 (s, 2H, CH_2), 3.77 (s, 3H, OCH_3), 3.73-3.71 (m, 2H, CH_2), 3.51 (t, 2H, *J* = 6.4, CH_2), 3.39-3.35 (m, 4H, 2 × CH_2), 2.16 (s, 3H, CH_3). HRMS *m/z* calcd for $C_{35}H_{37}N_{11}O_4F_2 + H^+$: 714.30708, obsd 714.30709.

Monomeric ligand 8b. Yield after column purification: 71 mg (93 µmol, 62%). LC-MS analysis: t_R 6.44 min (linear gradient 10 to 90% B in 14.5 min; *m/z*: 758.27 $[M + H]^+$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.64 (d, 1H, *J* = 4.8, CH Ar), 8.12 (br s, 1H, NH), 8.07 (t, 1H, *J* = 4.4, CH Ar), 7.72 – 7.68 (m, 2H, CH Trl, CH Ar), 7.55 (t, 1H, *J* = 6.4, CH Ar), 7.31 – 7.24 (m, 2H, 2 × CH Ar), 6.93 – 6.67 (m, 3H, 3 × CH Ar), 6.76 – 6.73 (m, 2H, 2 × CH Ar), 5.24 (s, 2H, CH_2), 4.50 – 4.46 (m, 4H, 2 × CH_2), 4.22 (t, 2H, *J* = 5.6, CH_2), 3.83 (t, 2H, *J* = 5.6, CH_2), 3.79 (s, 5H, OCH_3, CH_2), 3.59 (t, 2H, *J* = 5.2, CH_2), 3.41 (t, 2H, *J* = 6.8, CH_2), 3.38 – 3.32 (m, 6H, 2 × CH_2), 2.16 (s, 3H, CH_3). HRMS *m/z* calcd for $C_{37}H_{41}N_{11}O_5F_2 + H^+$: 758.33330, obsd 521.758.33322.

Monomeric ligand 8c. Yield after column purification: 76 mg (95 µmol, 63%). LC-MS analysis: t_R 6.54 min (linear gradient 10 to 90% B in 14.5 min; *m/z*: 802.33 $[M + H]^+$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.64 (d, 1H, *J* = 5.2, CH Ar), 8.01 (t, 1H, *J* = 4.4, CH Ar), 7.97 (t, 1H, *J* = 4.8, NH), 7.70 – 7.65 (m, 2H, CH Trl, CH Ar), 7.52 (t, 1H, *J* = 5.6, CH Ar), 7.31 – 7.24 (m, 2H, 2 × CH Ar), 6.93 – 6.86 (m, 3H, 3 × CH Ar), 6.76 – 6.73 (m, 2H, 2 × CH Ar), 5.25 (s, 2H, CH_2), 4.48 – 4.51 (m, 4H, 2 × CH_2), 4.22 (t, 2H, *J* = 7.5, CH_2), 3.84 (t, 2H, *J* = 4.8, CH_2), 3.79 (s, 3H, OCH_3), 3.64 – 3.60 (m, 6H, 3 × CH_2), 3.38 – 3.30 (m, 8H, 4 × CH_2), 3.21 (t, 2H, *J* = 5.6, CH_2), 2.16 (s, 3H, CH_3). HRMS *m/z* calcd for $C_{39}H_{45}N_{11}O_6F_2 + H^+$: 802.35951, obsd 802.35949.

Monomeric ligand 8d. Yield after column purification: 90 mg (106 µmol, 71%). LC-MS analysis: t_R 6.60 min (linear gradient 10 to 90% B in 14.5 min; *m/z*: 846.33 $[M + H]^+$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.65 (d, 1H, *J* = 5.7, CH Ar), 8.03 (t, 1H, *J* = 8.0, CH Ar), 7.83 (br s, 1H, NH), 7.70 (s, 1H, CH Trl), 7.65 (d, 1H, *J* = 7.6, CH Ar), 7.51 (t, 1H, *J* = 5.6, CH Ar), 7.35 – 7.22 (m, 2H, 2 × CH Ar), 6.93 – 6.84 (m, 3H, 3 × CH Ar), 6.82 – 6.68 (m, 2H, 2 × CH Ar), 5.25 (s, 2H, CH_2), 4.48 – 4.51 (m, 4H, 2 × CH_2), 4.16 (t, 2H, *J* = 6.0, CH_2), 3.84 (t, 2H, *J* = 5.2, CH_2), 3.79 (s, 3H, OCH_3), 3.69 – 3.57 (m, 10H, 5 × CH_2), 3.38 (t, 2H, *J* = 4.8, CH_2), 3.30 (t, 2H, *J* = 4.8, CH_2), 3.18 (t, 2H, *J* = 6.0, CH_2), 2.16 (s, 3H, CH_3). HRMS *m/z* calcd for $C_{41}H_{49}N_{11}O_7F_2 + H^+$: 846.38573, obsd 846.38548.

Monomeric ligand 8e. Yield after column purification: 54 mg (61 µmol, 40%). LC-MS analysis: t_R 6.63 min (linear gradient 10 to 90% B in 14.5 min; *m/z*: 890.40 $[M + H]^+$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.63 (d, 1H, *J* = 5.1, CH Ar), 8.15 (t, 1H, *J* = 5.3, CON*H*), 7.75 – 7.72 (m, 2H, CH Ar, CH Trl), 7.56 (t, 1H, *J* = 7.3, CH Ar), 7.35 – 7.22 (m, 2H, 2 × CH Ar), 6.92 – 6.84 (m, 3H, 3 × CH Ar), 6.76 – 6.73 (m, 2H, 2 × CH Ar), 5.23 (s, 2H, CH_2), 4.50 – 4.45 (m, 4H, 2 × CH_2), 4.21 (t, 2H, *J* = 6.6, CH_2), 3.83 (t, 2H, *J* = 5.2, CH_2), 3.79 (s, 3H, OCH_3), 3.69 – 3.56 (m, 14H, 6 × OCH_2), 3.51 (t, *J* = 4.8, 2H, CH_2), 3.42 – 3.34 (m, 6H, 3 × CH_2), 2.16 (s, 3H, CH_3). HRMS *m/z* calcd for $C_{43}H_{53}N_{11}O_8F_2 + H^+$: 890.41194, obsd 890.41168.

General method for the preparation of homodimeric ligands 9a-e.

A solution of the acetylene functionalized ligand **7** (24.0 mg, 40 μmol) and bis-azide spacer **17a, 17b, 17c, 17d** or **17e** (20 μmol) in a mixture of *t*BuOH/CH_3CN/H_2O (2/2/1; v/v/v, 800 μL) was degassed for 1h. Sodium ascorbate (1 eq 40 μL of a 1M solution in degassed H_2O) and $CuSO_4$ (0.2 eq 40 μL of a 0.2M solution in degassed H_2O) were added and the reaction mixture was stirred at 60 °C for until LC-MS showed complete conversion of the starting material (2-3 h). The mixture was filtered and the crude products were purified by preparative RP-HPLC (linear gradient of 3.0 CV; 37.5 to 45% B for **9a** and **9b**; 37.5 to 38.5% B for **9c**, **9d** and **9e**). Evaporation and lyophilization of the combined fractions furnished dimeric ligands **9a-e** as TFA salts as white amorphous powders.

Homodimeric ligand 9a. Yield after RP-HPLC purification: 9.4 mg (6.1 μmol, 30%). LC-MS analysis: t_R 6.70 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1315.60 $[M + H]^+$). ^1H NMR (400 MHz, $CDCl_3$) δ 8.61 (d, 2H, *J* = 4.8, 2 × CH Ar), 8.40 (br s, 2H, 2 × NH), 8.03 (t, 2H, *J* = 5.2, 2 × CH Ar), 7.64 (d, 2H, *J* = 7.8, 2 × CH Ar), 7.50 (t, 4H, *J* = 6.4, 4 × CH Ar), 7.30 – 7.26 (m, 6H, 4 × CH Ar, 2 × CH Trl), 6.97 – 6.82 (m, 6H, 6 × CH Ar), 6.79 – 6.70 (m, 6H, 4 × CH Ar), 5.23 (s, 4H, 2 × CH_2), 4.59 (s, 4H, 2 × CH_2), 4.33 (br s, 4H, 2 × CH_2), 4.18 (t, 4H, *J* = 5.6, 2 × CH_2), 3.77 (s, 6H, 2 × CH_3), 3.43 (t, 4H, *J* = 6.0, 2 × CH_2), 3.32 (t, 4H, *J* = 5.8, 2 × CH_2), 3.27 – 3.14 (m, 4H, 2 × CH_2), 2.17 (s, 6H, 2 × CH_3). HRMS *m/z* calcd for $C_{68}H_{70}N_{16}O_8F_4 + H^+$: 1315.55714, obsd 1315.55485.

Homodimeric ligand 9b. Yield after RP-HPLC purification: 8.2 mg (5.2 μmol, 26%). LC-MS analysis: t_R 6.71 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1359.73 $[M + H]^+$). ^1H NMR (400 MHz, $CDCl_3$) δ 8.67 (br s, 2H, 2 × NH), 8.61 (d, 2H, *J* = 6.0, 2 × CH Ar), 8.08 (t, 2H, *J* = 6.0, 2 × CH Ar), 7.76 (d, 2H, *J* = 7.6, 2 × CH Ar), 7.56 – 53 (m, 4H, 2 × CH Ar, 2 × CH Trl), 7.31 – 7.26 (m, 4H, 4 × CH Ar), 6.92 – 6.85 (m, 6H, 6 × CH Ar), 6.76 – 6.72 (m, 4H, 4 × CH Ar), 5.24 (s, 4H, 2 × CH_2), 4.48 (d, 4H, *J* = 4.4, 2 × CH_2), 4.34 (t, 4H, *J* = 4.8, 2 × CH_2), 4.28 (t, 4H, *J* = 5.2, 2 × Trl CH_2), 4.07 (br s, 4H, 2 × CH_2), 3.77 (s, 6H, 2 × CH_3), 3.72 – 3.64 (m, 8H, 2 × OCH_2, 2 × CH_2), 3.48 – 3.38 (m, 8H, 4 × CH_2), 2.15 (s, 6H, 2 × CH_3). HRMS *m/z* calcd for $C_{70}H_{74}N_{16}O_9F_4 + H^+$: 1359.58336, obsd 1359.58554.

Homodimeric ligand 9c. Yield after RP-HPLC purification: 7.2 mg (4.4 μmol, 22%). LC-MS analysis: t_R 6.72 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1403.73 $[M + H]^+$). ^1H NMR (400 MHz, $CDCl_3$) δ 8.60 (d, 2H, *J* = 6.0, 2 × CH Ar), 8.45 (br s, 2H, 2 × NH), 8.01 (t, 2H, *J* = 7.6, 2 × CH Ar), 7.66 – 7.64 (m, 4H, 2 × CH Ar, 2 × CH Trl), 7.49 (t, 2H, *J* = 6.0, 2 × CH Ar), 7.30 – 7.26 (m, 4H, 4 × CH Ar), 6.92 – 6.85 (m, 6H, 6 × CH Ar), 6.75 – 6.72 (m, 4H, 4 × CH Ar), 5.23 (s, 4H, 2 × CH_2), 4.45 (d, 4H, *J* = 6.0, 2 × CH_2), 4.39 (t, 4H, *J* = 4.4, 2 × CH_2), 4.21 (t, 4H, *J* = 5.6, 2 × CH_2), 3.84 (br s, 4H, 2 × CH_2), 3.77 (s, 6H, CH_2, 2 × CH_3), 3.74 – 3.69 (m, 4H, 2 × CH_2), 3.50 – 3.48 (m, 8H, 4 × CH_2), 3.36 – 3.31 (m, 8H, 4 × CH_2), 2.15 (s, 6H, 2 × CH_3). HRMS *m/z* calcd for $C_{72}H_{78}N_{16}O_{10}F_4 + H^+$: 1403.60957, obsd 1403.60730.

Homodimeric ligand 9d. Yield after RP-HPLC purification: 4.6 mg (2.8 μmol, 14%). LC-MS analysis: t_R 6.70 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1447.73 $[M + H]^+$). ^1H NMR (400 MHz, $CDCl_3$) δ 8.60 (d, *J* = 5.2, 2H, 2 × CH Ar), 8.40 (br s, 2H, 2 × NH), 8.05 (t, 2H, *J* = 8.0, 2 × CH Ar), 7.72 – 7.69 (m, 4H, 2 × CH Ar, 2 × CH Trl), 7.52 (t, 2H, *J* = 6.0, 2 × CH Ar), 7.30 – 7.26 (m, 4H, 4 × CH Ar), 6.92 – 6.85 (m, 6H, 6 × CH Ar), 6.75 – 6.72 (m, 4H, 4 × CH Ar), 5.22 (s, 4H, 2 × CH_2), 4.45 – 4.40 (m, 8H, 4 × CH_2), 4.23 (t, 4H, *J* = 5.6, 2 × CH_2), 3.87 (br s, 4H, 2 × CH_2), 3.80 (t, 4H, *J* = 4.8, 2 × CH_2), 3.77 (s, 6H, CH_2, 2 × CH_3), 3.70 (s, 4H, 2 × CH_2), 3.55 – 3.49 (m, 12H, 6 × CH_2), 3.36 – 3.35 (m, 4H, 4 × CH_2), 2.14 (s, 6H, 2 × CH_3). HRMS *m/z* calcd for $C_{74}H_{82}N_{16}O_{11}F_4 + 2H^+$: 724.32153, obsd 724.32119.

Homodimeric ligand 9e. Yield after RP-HPLC purification: 6.0 mg (3.5 μmol, 17%). LC-MS analysis: t_R 6.70 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1491.80 $[M + H]^+$). ^{1}H NMR (400 MHz, DMSO-*d*6) δ 8.96-8.79 (m, 2H, CON*H*), 8.52 (t, 2H, *J* = 4.7, 2 × CH Ar), 8.33 (s, 2H, 2 × NH), 7.95-7.86 (m, 4H, 2 × CH Ar, 2 × CH Trl), 7.48 – 7.37 (m, 2H, 2 × CH Ar), 7.33 (dt, 2H, *J* = 2.1, 7.8, 2 × CH Ar), 7.11 (dt, 2H, *J* = 2.0, 8.4, 2 × CH Ar), 6.98 – 6.91 (m, 2H, 2 × CH Ar), 6.77-6.70 (m, 4H, 4 × CH Ar), 5.24 (s, 4H, 2 × CH_2), 4.44 (dd, 4H, 2 × OCH_2), 4.38 (t, 4H, *J* = 4.8, 2 × OCH_2), 4.19 – 4.09 (m, 4H, 2 × CH_2), 4.00 – 3.87 (m, 4H, 2 × CH_2), 3.80 – 3.71 (m, 7H, OCH_3, 2 × OCH_2), 3.59 (t, 4H, *J* = 7.0, 2 × OCH_2), 3.55 – 3.42 (m, 8H, 4 × CH_2), 3.41 (t, 4H, *J* = 7.6, 2 × OCH_2), 3.34 – 3.25 (m, 4H, 2 × CH_2), 3.15 (dd, *J* = 7.6, 15.5, 2H, 2 × CH_2), 2.19 (s, 6H, 2 × CH_3). HRMS *m/z* calcd for $C_{76}H_{86}N_{16}O_{12}F_4$ + $2H^+$: 746.33464, obsd 746.33456.

General method for the preparation of heterodimeric ligands 10a-e and 11a-e.
A solution of the monomeric ligand **8a, 8b, 8c, 8d** or **8e** (20 μmol) and acetylene derivatives **3** or **4** (0.25 μmol) in a mixture of *t*BuOH/CH_3CN/H_2O (2/2/1; v/v/v, 1 mL) was degassed for 1h. Sodium ascorbate (1 eq. 20 μL of a 1M solution in degassed H_2O) and $CuSO_4$ (0.2 eq. 20 μL of a 0.2M solution in degassed H_2O) were added and the reaction mixture was stirred at 60°C until LC-MS showed complete conversion of the starting material (2-3h). The mixture was filtered and the crude products were purified by preparative RP-HPLC (linear gradient of 3.0 CV; 40 to 45% B for **10a-e** and 42.5 to 45% B for **11a-e**). Evaporation and lyophilization of the combined fractions furnished hetero-dimeric ligands as TFA salts as white amorphous powders.

Heterodimeric ligand 10a. Yield after RP-HPLC purification: 5.9 mg (3.5 μmol,17%). LC-MS analysis: t_R 6.91 min (linear gradient 10 to 90% B in 14.5 min; *m/z*: 1352.33 $[M + H]^+$). ^{1}H NMR (600 MHz, DMSO-*d*6) δ 9.03 (s, 1H, =CH), 8.89 (br s, 2H, N*H*CONH, NH^+ TFA^-), 8.71 (br s, 1H, NH^+ TFA^-), 8.50 (d, 1H, *J* = 4.4), 7.89 (s, 3H, 2 × =C*H* Tzl, NH^+ TFA^-), 7.53 (dd, 4H, *J* = 8.0), 7.48 (t, 1H, *J* = 7.5), 7.45 – 7.38 (m, 9H, 9 × CH Ar), 7.31 (t, 1H, *J* = 7.9), 7.18 (dd, 2H, *J* = 6.8, 9.6, 2 × CH Ar), 7.10 (dd, 2H, *J* = 7.3, 9.3, 2 × CH Ar), 6.93 (d, 1H, *J* = 8.9), 6.76-6.71 (m, 4H, 2 × CH Ar, CON*H*, NHCON*H*), 5.65 (s, 2H, CH_2), 5.22 (s, 2H, CH_2), 5.10 (d, 1H, *J* = 12.1, C*H*H), 4.82 – 4.77 (m, 5H, 2 × CH_2 Trl, CH*H*), 4.56 (d, 1H, *J* = 12.6, C*H*H), 4.37 – 4.29 (m, 7H, C*H*$_2$$CH_3$, 2 × NHC*H*$_2$ Trl, CH*H*), 4.13 (s, 2H, CH_2), 3.92 – 3.87 (m, 2H, CH_2), 3.77 (s, 3H, OCH_3), 3.45-3.35 (m, 2H, CH_2), 3.27 (br s, 2H, CH_2), 3.18 – 3.12 (m, 2H, CH_2), 2.50 (s, 3H, NC*H*$_3$), 2.18 (s, 3H, =CC*H*$_3$), 1.34 (t, 3H, *J* = 7.1, CH_2C*H*$_3$). HRMS *m/z* calcd for $C_{70}H_{69}N_{17}O_8F_4$ + $2H^+$: 676.77983, obsd 676.78005.

Heterodimeric ligand 10b. Yield after RP-HPLC purification: 5.7 mg (3.3 μmol, 16%). LC-MS analysis: t_R 6.89 min (linear gradient 10 to 90% B in 14.5 min; *m/z*: 1396.27 $[M + H]^+$). ^{1}H NMR (600 MHz, DMSO-*d*6) δ 9.03 (s, 1H, =CH), 8.89 (s, 1H, N*H*CONH), 8.83 (br s, 1H, NH^+ TFA^-), 8.71 (br s, 1H, NH^+ TFA^-), 8.50 (d, 1H, *J* = 4.1), 7.85 (s, 1H, =C*H* Tzl), 7.83 (s, 2H, =C*H* Tzl, NH^+ TFA^-), 7.53 (dd, 4H, *J* = 8.0), 7.48 (t, 1H, *J* = 7.5), 7.42 – 7.38 (m, 9H, 9 × CH Ar), 7.31 (t, 1H, *J* = 7.8), 7.17 (t, 2H, *J* = 7.8, 2 × CH Ar), 7.09 (t, 2H, *J* = 7.8, 2 × CH Ar), 6.92 (d, 1H, *J* = 7.8), 6.76-6.71 (m, 4H, 2 × CH Ar, CON*H*, NHCON*H*), 5.65 (s, 2H, CH_2), 5.22 (s, 2H, CH_2), 5.10 (d, 1H, *J* = 13.2, C*H*H), 4.78 (d, 1H, *J* = 12.3, CH*H*), 4.56 (d, 1H, *J* = 7.3, C*H*H), 4.49 (t, 2H, *J* = 4.9, CH_2 Trl), 4.46 (t, 2H, *J* = 4.9, CH_2 Tlz), 4.37 – 4.32 (m, 7H, C*H*$_2$$CH_3$, 2 × NHC*H*$_2$ Trl, CH*H*), 4.11 (s, 2H, CH_2), 3.76 – 3.73 (m, 7H, OCH_3, 2 × OCH_2), 3.45 – 3.35 (m, 2H, CH_2), 3.30 – 3.20 (m, 2H, CH_2), 3.13 – 3.08 (m, 2H, CH_2), 2.50 (s, 3H, NC*H*$_3$), 2.17 (s, 3H, =CC*H*$_3$), 1.34 (t, 3H, *J* = 7.2, CH_2C*H*$_3$). HRMS *m/z* calcd for $C_{72}H_{73}N_{17}O_9F_4$ + $2H^+$: 698.79294, obsd 698.79294.

Heterodimeric ligand 10c. Yield after RP-HPLC purification: 3.3 mg (1.9 μmol, 9%). LC-MS analysis: t_R 6.92 min (linear gradient 10 to 90% B in 14.5 min; *m/z*: 1440.33 $[M + H]^+$). ^{1}H NMR (600 MHz, DMSO-*d*6) δ 9.03 (s, 1H, =CH), 8.85 (s, 1H, N*H*CONH), 8.83 (br s, 1H, NH^+ TFA^-), 8.70 (br s, 1H, NH^+ TFA^-), 8.48 (d, 1H, *J* = 4.1), 7.92

(s, 1H, =C*H* Tzl), 7.89 (s, 1H, =C*H* Tzl), 7.81 (br s, 1H, NH^+ TFA^-), 7.53 (dd, 4H, *J* = 8.0), 7.46 (t, 1H, *J* = 7.2), 7.42 – 7.35 (m, 9H, 9 × CH Ar), 7.31 (t, 1H, *J* = 7.8), 7.17 (t, 2H, *J* = 7.8, 2 × CH Ar), 7.09 (t, 2H, *J* = 7.8, 2 × CH Ar), 6.92 (d, 1H, *J* = 9.0), 6.73 – 6.71 (m, 4H, 2 × CH Ar, CON*H*, NHCON*H*), 5.65 (s, 2H, CH_2), 5.22 (s, 2H, CH_2), 5.10 (br d, 1H, *J* = 11.3, C*H*H), 4.78 (d, *J* = 9.7, CH*H*), 4.56 (d, 1H, *J* = 11.1, C*H*H), 4.49 (t, 2H, *J* = 5.0, CH_2 Trl), 4.40 (t, 2H, *J* = 4.9, CH_2 Trl), 4.37 – 4.32 (m, 7H, C$H_2$$CH_3$, 2 × NHC$H_2$ Trl, CH*H*), 4.17 – 4.07 (m, 2H, CH_2), 3.76 – 3.73 (m, 5H, OCH_3, OCH_2), 3.70 (t, 2H, *J* = 4.9, OCH_2), 3.56 (s, 4H, 2 × OCH_2), 3.45 – 3.35 (m, 2H, CH_2), 3.25 – 3.15 (m, 2H, CH_2), 3.13 – 3.08 (m, 2H, CH_2), 2.50 (s, 3H, NCH_3), 2.17 (s, 3H, =CCH_3), 1.34 (t, 3H, *J* = 7.2, CH_2CH_3). HRMS *m/z* calcd for $C_{74}H_{77}N_{17}O_{10}F_4$ + $2H^+$: 720.80605, obsd 720.80578.

Heterodimeric ligand 10d. Yield after RP-HPLC purification: 6.0 mg (3.3 μmol, 16%). LC-MS analysis: t_R 6.92 min (linear gradient 10 to 90% B in 14.5 min; *m/z*: 1484.27 $[M + H]^+$). ^{1}H NMR (600 MHz, DMSO-*d*6) δ 9.03 (s, 1H, =CH), 8.89 (s, 1H, N*H*CONH), 8.86 (br s, 1H, NH^+ TFA^-), 8.76 (br s, 1H, NH^+ TFA^-), 8.49 (d, 1H, *J* = 3.6), 7.94 (s, 1H, =C*H* Tzl), 7.91 (s, 1H, =C*H* Tzl), 7.89 (br s, 1H, NH^+ TFA^-), 7.58 (dd, 4H, *J* = 8.5), 7.51 (t, 1H, *J* = 7.6), 7.46 – 7.35 (m, 9H, 9 × CH Ar), 7.32 (t, 1H, *J* = 7.9), 7.18 (t, 2H, *J* = 8.0, 2 × CH Ar), 7.10 (t, 2H, *J* = 8.2, 2 × CH Ar), 6.93 (d, 1H, *J* = 8.7), 6.77 (t, 1H, *J* = 5.3, CON*H*), 6.74 – 6.70 (m, 3H, 2 × CH Ar, NHCON*H*), 5.66 (s, 2H, CH_2), 5.23 (s, 2H, CH_2), 5.14 (br d, 1H, *J* = 13.8, C*H*H), 4.83 (br d, 1H, *J* = 12.8, CH*H*), 4.60 (br d, 1H, *J* = 11.8, C*H*H), 4.50 (t, 2H, *J* = 5.0, CH_2 Trl), 4.42 (t, 2H, *J* = 4.9, CH_2 Trl), 4.39 – 4.29 (m, 7H, C$H_2$$CH_3$, 2 × NHC$H_2$ Trl, CH*H*), 4.15 – 4.08 (m, 2H, CH_2), 3.81 (t, 2H, *J* = 5.0, OCH_2), 3.76 – 3.71 (m, 5H, OCH_3, OCH_2), 3.58 (s, 4H, 2 × OCH_2), 3.51 – 3.42 (m, 6H, 2 × OCH_2, CH_2), 3.28 – 3.19 (m, 2H, CH_2), 3.15 – 3.06 (m, 2H, CH_2), 2.51 (s, 3H, NCH_3), 2.18 (s, 3H, =CCH_3), 1.35 (t, *J* = 7.1, 3H, CH_2CH_3). HRMS *m/z* calcd for $C_{76}H_{81}N_{17}O_{11}F_4$ + $2H^+$: 742.81916, obsd 742.81938.

Heterodimeric ligand 10e. Yield after RP-HPLC purification: 5.2 mg (2.8 μmol, 14%). LC-MS analysis: t_R 6.93 min (linear gradient 10 to 90% B in 14.5 min; *m/z*: 1529.07 $[M + H]^+$). ^{1}H NMR (600 MHz, DMSO-*d*6) δ 9.03 (s, 1H, =CH), 8.86 (s, 2H, N*H*CONH, NH^+ TFA^-), 8.71 (br s, 1H, NH^+ TFA^-), 8.49 (d, 1H, *J* = 2.6), 7.95 (s, 1H, =C*H* Tzl), 7.91 (s, 1H, =C*H* Tzl), 7.83 (br s, 1H, NH^+ TFA^-), 7.54 (dd, 4H, *J* = 8.5), 7.48 (t, 1H, *J* = 7.6), 7.46 – 7.35 (m, 9H, 9 × CH Ar), 7.32 (t, 1H, *J* = 7.9), 7.18 (t, 2H, *J* = 8.2, 2 × CH Ar), 7.10 (m, 3H, 2 × CH Ar, CON*H*), 6.93 (d, 1H, *J* = 9.1), 6.74 – 6.70 (m, 3H, 2 × CH Ar, NHCON*H*), 5.66 (s, 2H, CH_2), 5.23 (s, 2H, CH_2), 5.11 (br d, 1H, *J* = 12.6, C*H*H), 4.83 (br d, 1H, *J* = 10.9, CH*H*), 4.60 (br d, 1H, *J* = 12.5, C*H*H), 4.50 (t, 2H, *J* = 5.1, CH_2 Trl), 4.42 (t, 2H, *J* = 5.1, CH_2 Trl), 4.39 – 4.29 (m, 7H, C$H_2$$CH_3$, 2 × NHC$H_2$ Trl, CH*H*), 4.15 – 4.08 (m, 2H, CH_2), 3.81 (t, 2H, *J* = 4.8, OCH_2), 3.76 – 3.71 (m, 5H, OCH_3, OCH_2), 3.58 (s, 4H, 2 × OCH_2), 3.51 – 3.42 (m, 6H, 2 × OCH_2, CH_2), 3.28 – 3.19 (m, 2H, CH_2), 3.15 – 3.06 (m, 2H, CH_2), 2.51 (s, 3H, NCH_3), 2.18 (s, 3H, =CCH_3), 1.35 (t, 3H, *J* = 7.1, CH_2CH_3). HRMS *m/z* calcd for $C_{78}H_{85}N_{17}O_{12}F_4$ + $2H^+$: 764.83226, obsd 764.83262.

Heterodimeric ligand 11a. Yield after RP-HPLC purification: 8.0 mg (4.5 μmol, 23%). LC-MS analysis: t_R 7.07 min (linear gradient 10 to 90% B in 14.5 min; *m/z*: 1423.47 $[M + H]^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 8.96 (s, 1H, *H*C=), 8.89 – 8.60 (br s, 4H, 2 × NH^+ TFA^-, NHCON*H*, CON*H*), 8.50 (d, 1H, *J* = 4.2, CH Ar), 7.87 (m, 2H, CH Ar, =C*H* Tzl), 7.74 (s, 1H, =C*H* Tzl), 7.56 – 7.48 (m, 4H, 4 × CH Ar), 7.46 – 7.35 (m, 4H, 4 × CH Ar), 7.35 – 7.29 (app. t, 3H, *J* = 7.9 3 × CH Ar), 7.28 – 7.20 (m, 3H, 3 × CH Ar), 7.16 (t, 2H, *J* = 8.2, 2 × CH Ar), 7.10 (t, 2H, *J* = 8.3, 2 × CH Ar), 6.93 (d, *J* = 9.1, 1H, CH Ar), 6.74 – 6.68 (m, 2H, 2 × CH Ar), 6.28 (br s, 1H, N*H*CONH), 5.60 (s, 2H, CH_2), 5.23 (s, 2H, CH_2), 4.78 (br s, 2H, CH_2 Ar), 4.52 (br s, 2H, CH_2), 4.37 – 4.27 (m, 6H, 2 × CH_2, COOC$H_2$$CH_3$), 4.15 – 4.09 (m, 4H, 2 × CH_2), 4.08 – 3.97 (m, 2H, CH_2), 3.93 – 3.81 (br s, 2H, NCH_2CONH), 3.74 (s, 3H, OCH_3), 3.49 – 3.42 (br s, 2H, CH_2), 3.30 – 3.19 (br s, 2H, CH_2), 3.18 – 3.12 (m, 4H, CH_2, CONHC$H_2$$CH_3$), 2.18 (s, 3H, =C$CH_3$), 1.33 (t, 3H, *J* = 7.1, CH_2CH_3), 1.06 (t, 3H, , *J* = 7.1, CH_2CH_3). HRMS *m/z* calcd for $C_{73}H_{74}N_{18}O_9F_4$ + $2H^+$: 712.29839, obsd 712.29861.

Heterodimeric ligand 11b. Yield after RP-HPLC purification: 10.9 mg (6.6 μmol, 33%). LC-MS analysis: t_R 7.12 min (linear gradient 10 to 90% B in 14.5 min; *m/z*: 1467.47 $[M + H]^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 8.96 (s, 1H, *H*C=), 8.89 – 8.78 (br s, 4H, 2 × NH^+ TFA^-, NHCON*H*, CON*H*), 8.50 (d, 1H, *J* = 4.6, CH Ar), 7.89 (t, 1H, *J* = 7.5, CH Ar), 7.83 (s, 1H, =C*H* Tzl), 7.74 (s, 1H, =C*H* Tzl), 7.55 – 7.50 (m, 4H, 4 × CH Ar), 7.47 – 7.37 (m, 4H, 4 × CH Ar), 7.36 – 7.29 (m, 3H, 3 × CH Ar), 7.28 – 7.19 (m, 3H, 3 × CH Ar), 7.17 (t, 2H, *J* = 8.2, 2 × CH Ar), 7.10 (t, 2H, *J* = 8.2, 2 × CH Ar), 6.92 (d, *J* = 9.1, 1H, CH Ar), 6.74 – 6.68 (m, 2H, 2 × CH Ar), 6.36 (br s, 1H, N*H*CONH), 5.60 (s, 2H, CH_2), 5.22 (s, 2H, CH_2), 4.89 (br s, 2H, CH_2 Ar), 4.50 (br s, 2H, CH_2), 4.42 (t, 2H, *J* = 4.9, OCH_2CH_2 Trl), 4.39 (t, 2H, *J* = 4.9, OCH_2CH_2 Trl), 4.35 (d, 2H, *J* = 5.1, CH_2), 4.29 (q, 4H, *J* = 7.0, $COOCH_2CH_3$), 4.17 (br t, 2H, *J* = 5.7, CH_2), 4.09 (s, 2H, CH_2), 3.93 (s, 2H, CH_2), 3.88 (br s, 2H, NCH_2CONH), 3.76 – 3.69 (m, 7H, OCH_3, 2 × OCH_2), 3.48 (s, 2H, CH_2), 3.30 (s, 2H, CH_2), 3.17 – 3.09 (m, 4H, CH_2, $CONHCH_2CH_3$), 2.18 (s, 3H, $=CCH_3$), 1.33 (t, 3H, *J* = 7.1, CH_2CH_3), 1.06 (t, 3H, *J* = 7.2, CH_2CH_3). HRMS *m/z* calcd for $C_{75}H_{78}N_{18}O_{10}F_4 + 2H^+$: 734.31150, obsd 734.31160.

Heterodimeric ligand 11c. Yield after RP-HPLC purification: 5.5 mg (3.0 μmol, 15%). LC-MS analysis: t_R 7.12 min (linear gradient 10 to 90% B in 14.5 min; *m/z*: 1511.47 $[M + H]^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 8.94 (s, 1H, *H*C=), 8.88 – 8.63 (br s, 4H, 2 × NH^+ TFA^-, NHCON*H*, CON*H*), 8.49 (d, 1H, *J* = 4.2, CH Ar), 7.89 (s, 1H, =C*H* Tzl), 7.83 (br t, 1H, *J* = 6.1, CH Ar), 7.80 (s, 1H, =C*H* Tzl), 7.57 – 7.48 (m, 4H, 4 × CH Ar), 7.47 – 7.35 (m, 4H, 4 × CH Ar), 7.29 (app. t, 3H, *J* = 7.9, 3 × CH Ar), 7.28 – 7.21 (m, 3H, 3 × CH Ar), 7.17 (t, 2H, *J* = 8.1, 2 × CH Ar), 7.13 (t, 2H, *J* = 8.2, 2 × CH Ar), 6.93 (d, *J* = 9.0, 1H, CH Ar), 6.75 – 6.70 (m, 2H, 2 × CH Ar), 6.28 (br s, 1H, N*H*CONH), 5.60 (s, 2H, CH_2), 5.23 (s, 2H, CH_2), 5.04 – 4.72 (br s, 2H, CH_2 Ar), 4.69 – 4.49 (br s, 2H, CH_2), 4.44 (t, 2H, *J* = 5.0, OCH_2CH_2 Trl), 4.39 (t, 2H, *J* = 5.0, OCH_2CH_2 Trl), 4.36 (d, 2H, *J* = 5.0, CH_2), 4.29 (q, 4H, *J* = 7.0, $COOCH_2CH_3$), 4.11 (br s, 4H, 2 × CH_2), 3.88 (br s, 2H, NCH_2CONH), 3.75 (s, 3H, OCH_3), 3.71 (t, 2H, *J* = 5.1, 2 × OCH_2), 3.68 (t, 2H, *J* = 5.1, 2 × OCH_2), 3.47 – 3.44 (br s, 2H, CH_2), 3.44 – 3.39 (br s, 4H, 2 × OCH_2), 3.28 – 3.19 (s, 2H, CH_2), 3.16 – 3.08 (m, 4H, CH_2, $CONHCH_2CH_3$), 2.18 (s, 3H, $=CCH_3$), 1.33 (t, 3H, *J* = 7.1, CH_2CH_3), 1.07 (t, 3H, *J* = 7.2, CH_2CH_3). HRMS *m/z* calcd for $C_{77}H_{82}N_{18}O_{11}F_4 + 2H^+$: 756.32461, obsd 756.32478.

Heterodimeric ligand 11d. Yield after RP-HPLC purification: 6.6 mg (3.5 μmol, 17%). LC-MS analysis: t_R 7.09 min (linear gradient 10 to 90% B in 14.5 min; *m/z*: 1555.47 $[M + H]^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 9.02 – 8.63 (m, 5H, *H*C=, 2 × NH^+ TFA^-, NHCON*H*, CON*H*), 8.49 (d, 1H, *J* = 4.3, CH Ar), 7.90 (s, 1H, =C*H* Tzl), 7.88 – 7.85 (m, 1H, CH Ar), 7.81 (s, 1H, =C*H* Tzl), 7.58 – 7.49 (m, 4H, 4 × CH Ar), 7.49 – 7.35 (m, 4H, 4 × CH Ar), 7.32 (app. t, 3H, *J* = 7.9, 3 × CH Ar), 7.26 – 7.19 (m, 3H, 3 × CH Ar), 7.17 (t, 2H, *J* = 8.3, 2 × CH Ar), 7.13 (t, 2H, *J* = 8.3, 2 × CH Ar), 6.93 (d, 1H, *J* = 8.3, CH Ar), 6.75 – 6.69 (m, 2H, 2 × CH Ar), 6.24 (br s, 1H, N*H*CONH), 5.61 (s, 2H, CH_2), 5.26 – 5.20 (br s, 2H, CH_2), 5.11 – 4.69 (br s, 2H, CH_2 Ar), 4.69 – 4.49 (br s, 2H, CH_2), 4.46 (t, 2H, *J* = 5.0, OCH_2CH_2 Trl), 4.42 (t, 2H, *J* = 5.0, OCH_2CH_2 Trl), 4.36 (d, 2H, *J* = 5.0, CH_2), 4.30 (q, 4H, *J* = 6.7, $COOCH_2CH_3$), 4.19 – 4.02 (br s, 4H, 2 × CH_2), 3.97 – 3.81 (br s, 2H, NCH_2CONH), 3.78 – 3.71 (s, 7H, OCH_3, 2 × OCH_2), 3.45 – 3.21 (m, 10H, 4 × OCH_2, CH_2), 3.30 – 3.21 (br s, 2H, CH_2), 3.16 – 3.05 (m, 4H, CH_2, $CONHCH_2CH_3$), 2.18 (s, 3H, $=CCH_3$), 1.33 (t, 3H, *J* = 7.0, CH_2CH_3), 1.07 (t, 3H, *J* = 7.1, CH_2CH_3). HRMS *m/z* calcd for $C_{79}H_{86}N_{18}O_{12}F_4 + 2H^+$: 778.33771, obsd 778.33811.

Heterodimeric ligand 11e. Yield after RP-HPLC purification: 8.9 mg (4.6 μmol, 23%). LC-MS analysis: t_R 7.08 min (linear gradient 10 to 90% B in 14.5 min; *m/z*: 1599.53 $[M + H]^+$). ^{1}H NMR (500 MHz, DMSO-*d*6) δ 9.03 – 8.67 (m, 5H, *H*C=, 2 × NH^+ TFA^-, NHCON*H*, CON*H*), 8.50 (d, 1H, *J* = 4.2, CH Ar), 7.92 (s, 1H, =C*H* Tzl), 7.87 (br t, 1H, *J* = 7.0, CH Ar), 7.82 (s, 1H, =C*H* Tzl), 7.57 – 7.49 (m, 4H, 4 × CH Ar), 7.48 – 7.35 (m, 4H, 4 × CH Ar), 7.32 (app. t, 3H, *J* = 7.9, 3 × CH Ar), 7.28 – 7.21 (m, 3H, 3 × CH Ar), 7.17 (t, 2H, *J* = 8.1, 2 × CH Ar), 7.11 (t, 2H, *J* = 8.2, 2 × CH Ar), 6.94 (d, *J* = 9.1, 1H, CH Ar), 6.74 – 6.70 (m, 2H, 2 × CH Ar), 6.28 (br s, 1H, N*H*CONH), 5.61 (s, 2H,

CH_2), 5.23 (s, 2H, CH_2), 5.12 – 4.69 (br s, 2H, CH_2 Ar), 4.66 – 4.51 (br s, 2H, CH_2), 4.46 (t, 2H, J = 4.7, OCH_2CH_2 Trl), 4.42 (t, 2H, J = 5.1, OCH_2CH_2 Trl), 4.37 (d, 2H, J = 4.9, CH_2), 4.30 (q, 4H, J = 7.0, $COOCH_2CH_3$), 4.18 – 4.04 (m, 4H, 2 × CH_2), 3.99 – 3.82 (br s, 2H, NCH_2CONH), 3.78 – 3.71 (s, 7H, OCH_3, 2 × OCH_2), 3.52 – 3.38 (m, 14H, 6 × OCH_2, CH_2), 3.31 – 3.22 (br s, 2H, CH_2), 3.16 – 3.09 (m, 4H, CH_2, $CONHCH_2CH_3$), 2.18 (s, 3H, $=CCH_3$), 1.33 (t, 3H, J = 7.1, CH_2CH_3), 1.07 (t, 3H, J = 7.2, CH_2CH_3). HRMS m/z calcd for $C_{81}H_{90}N_{18}O_{13}F_4 + 2H^+$: 800.35082, obsd 800.35133.

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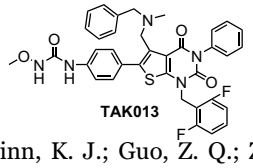

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Chapter 5

Discovery of selective LHR agonists by the bivalent ligand method

Introduction

The glycoprotein hormone receptors (GpHRs)[1,2] are part of a large family of G protein coupled receptors (GPCRs) that are distinguished by the nature of their endogenous ligands, the glycoprotein hormones. Three distinct GpHRs exist in man, namely the luteinizing hormone/choriogonadotropin receptor (LH/CGR),[3] the follicle-stimulating hormone receptor (FSHR)[4] and the thyroid-stimulating hormone receptor (TSHR).[5] The first two are key mediators in the human reproduction system whereas the latter controls endocrine production of the thyroid gland. The three GpHRs are highly homologous in their seven transmembrane α-helical part (the domain characteristic for the GPCR superfamily) and diverge in their extracellular domains. Although these domains fall into the so-called large N-terminal leucine rich repeat (LRR) category, they are differentiated such that each GpHR binds specifically and with high affinity to its glycoprotein hormone counterpart. There are four glycoprotein hormones described to date, each composed of an identical α-subunit and a unique β-subunit. Luteinizing hormone (LH) and human chorionic gonadotropin (hCG) both bind and agonize the LHR, follicle-stimulating

hormone (FSH) activates the FSHR and thyroid-stimulating hormone (TSH) induces TSHR signaling. For all receptor/hormone pairs, the combination of the unique β-subunit in the glycoprotein in combination with the nature of the LRR domain of the receptor is at the basis of selective ligand/receptor binding. From a therapeutic point of view, the ability to influence (agonize or antagonize) a specific GPCR with a small molecule having pharmacologically favorable properties is a major research objective. In endogenous events revolving around human reproduction both LHR and FSHR are often simultaneously activated, but selective therapeutic control in stimulation of these receptors is currently only possible by making use of their unique glycoprotein ligands.

Most effective small molecule GpHR modulators described to date are thought to exert their biological activity by binding to the seven-helical transmembrane region. This holds true also for the highly potent LMW LHR agonists Org 41841 (**1**)[6] and Org 43553 (**2**)[7-9] that are at the basis of the present study. Although potent LHR agonists, neither **1** nor **2** is capable of displacing ^{125}I-labeled hCG, suggesting a distinct binding site for these small molecule modulators. Molecular pharmacology studies making use of specifically mutated LHRs in combination with signal transduction experiments unambiguously point towards an allosteric binding site for these small molecule agonists, which most likely is located within the LHR transmembrane region.[7,8,10,11] Interestingly, compound **2** also shows activity on the FSHR, albeit with a lower potency.[9]

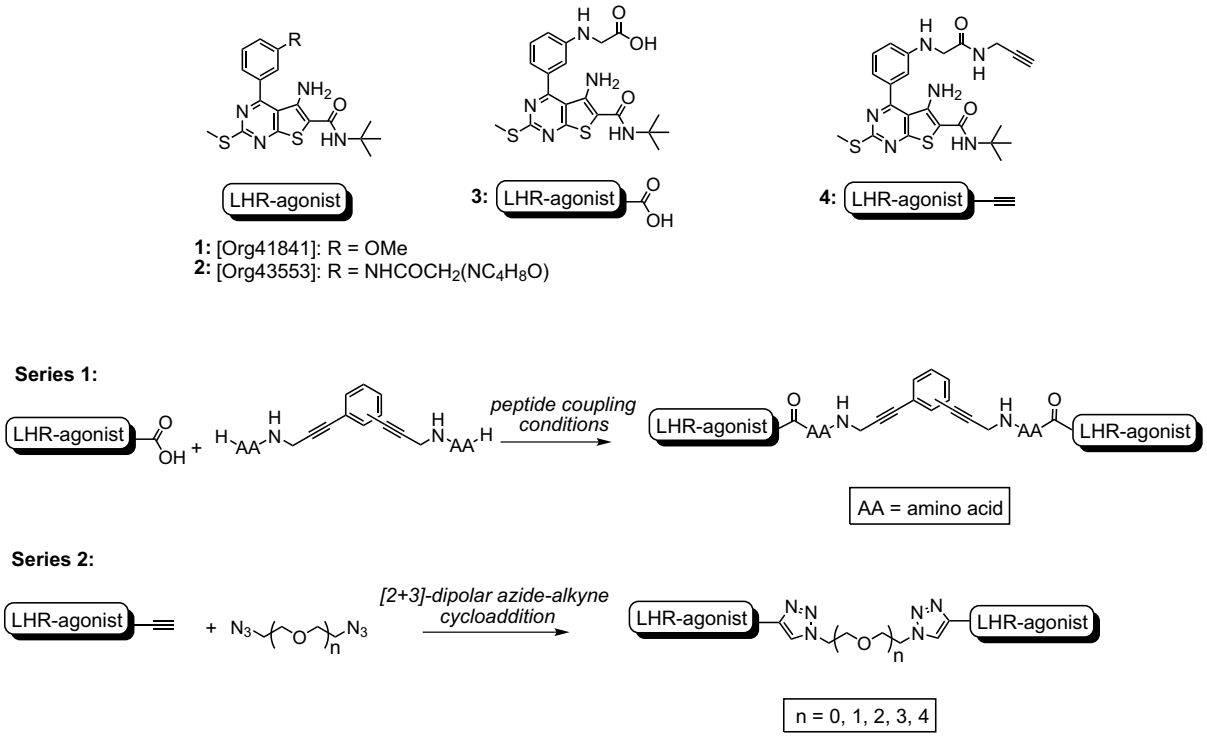
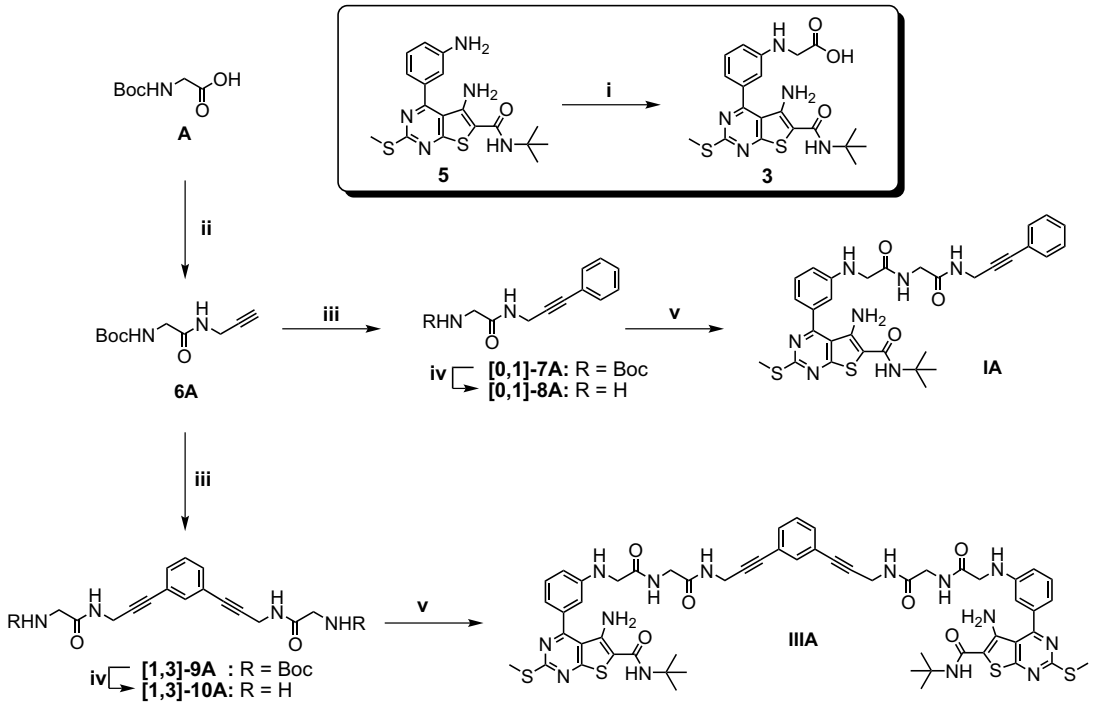


Figure 1. General features of the prepared compounds based on spacers containing a rigid benzene core (series 1) and flexible ethylene glycol spacers (series 2).

The aim of the here presented study is to establish whether improved selectivity for either the LHR or the FSHR can be achieved by chemical modifications of lead structure **2**. Literature precedents that GPCR (subtype-)specific ligands can be developed by dimerization of aspecific ligands.[12-14] This Chapter describes the development of two series of dimeric ligands (Figure 1) based on the parent LHR agonist **2**, modified at the aniline function to equip the monomer with a carboxylate (as in **3**) or an acetylene (as in **4**) handle. Compound **3** serves as a starting point for the library depicted in series 1 that is based on a rigid aryl linker system. Compound **4** is incorporated in the series 2 library, which is characterized by a flexible poly-ethylene glycol type linker system.

Results and discussion

A representative example of the synthetic route towards the compounds of series 1, bearing a substituted phenyl-core, is outlined in Scheme 1. Compound **5** (synthesized as reported)[9] was functionalized by a reductive alkylation with oxoacetic acid and sodium cyanoborohydride in a methanolic acetic acid/sodium acetate solution to give carboxylate **3**. Boc protected amine **[0,1]-7A** and diamine **[1,3]-9A** were prepared as described.[15] Cleavage of the *N*-Boc-group in **[0,1]-7A** (TFA/DCM) and subsequent condensation of **[0,1]-8A** with **3** under standard peptide condensation conditions (BOP, DiPEA, DMF) yielded compounds **IA.**



Scheme 1. Representative route for the synthesis of monomeric and dimeric compounds of series 1. *Reagents and conditions*: *i.* oxoacetic acid monohydrate, $NaCNBH_3$, HOAc/NaOAc, MeOH, 5d, 95%; *ii.* isobutyl chloroformate, *N*-methyl morpholine, propargylamine, DCM, -20 °C to rt, 18 h, 76%; *iii.* iodobenzene *or* 1,3-diiodobenzene, CuI, $Pd(PPh_3)_4$, pyrrolidine, DMF, 18 h, 66% for **[0,1]-7A**, 99% for **[1,3]-9A**; *iv.* TFA/DCM; 1/1; v/v, 1% TIS, 18 h, HPLC purification; *v.* pharmacophore **3**, BOP, DiPEA, DMF, 18 h, HPLC purification.

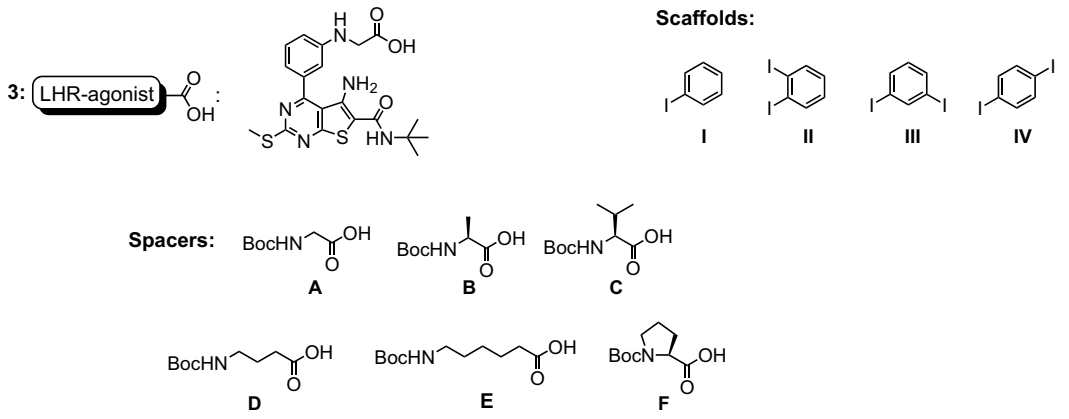
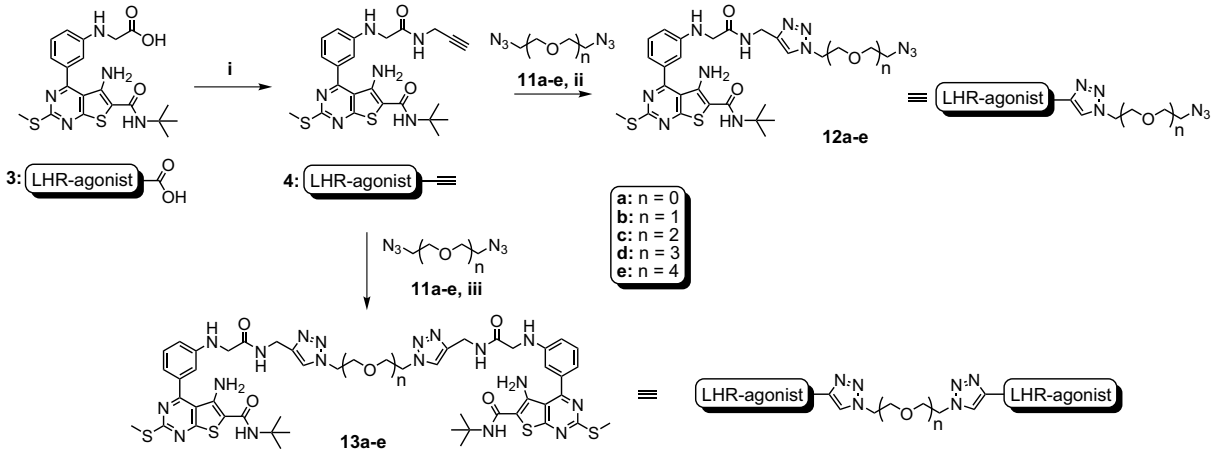


Figure 2. Aryl iodides and amino acids employed in the construction of series 1.

N-Boc-diamine **[1,3]-9A** was obtained by reacting two equivalents of propargyl derivative **6A** with 1,3-diiodobenzene. Boc-deprotection and condensation with **3** using the same conditions as described above yielded **IIIA**. In this fashion a 6 × 4 library was constructed that varies in substitution patterns of the benzene ring and the amino acids employed (depicted in Figure 2). All prepared compounds in this series are shown in Figure 3.

The dimeric ligands of series 2, bearing a flexible spacer, were obtained by a copper(I) catalyzed Huisgen [2+3]-cycloaddition of acetylene **4** to a set of azide-functionalized ethylene glycol spacers (**11a-e**, Scheme 2).[16] Compound **4** was obtained by condensation of **3** with propargylamine (BOP, DiPEA, DMF) in 86% yield. Addition of a 10-fold excess of diazide spacer to **4** in the presence of copper sulfate and sodium ascorbate in an acetonitrile/*tert*-butanol/water mixture gave mainly monomeric compounds **12a-e**. Dimeric compounds **13a-e** were obtained by adding two equivalents of **4** to diazide spacers **11a-e**.



Scheme 2. Synthesis of monomeric ligands **12a-e** and dimeric ligands **13a-e** containing flexible ethyleneglycol spacers (series 2). *Reagents and conditions*: *i*. propargylamine, BOP, DiPEA, DMF, 18 h, 95%; *ii*. 10 eq **11a-e**, 1 eq $CuSO_4$, 5 eq sodium ascorbate, *t*BuOH/CH_3CN/H_2O; 2/2/1; v/v/v, 60 °C, 2 h; *iii*. 0.5 eq. **11a-e**, 1 eq $CuSO_4$, 5 eq sodium ascorbate, *t*BuOH/CH_3CN/H_2O; 2/2/1; v/v/v, 60 °C, 2 h.

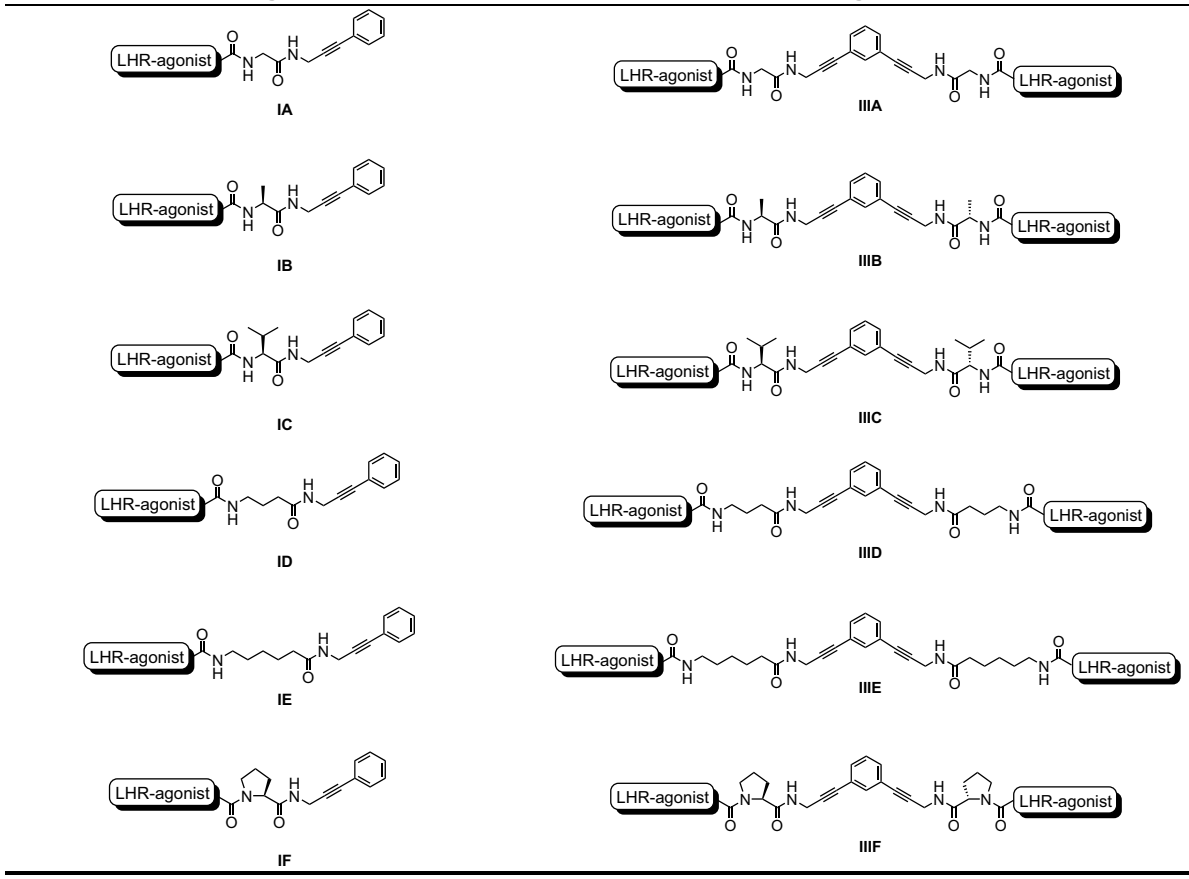


Figure 3. Stucture of monomeric ligands **IA-F** and dimeric ligands **IIA-F**, **IIIA-F** and **IVA-F**.

The compounds were assayed on their functional activity on the LHR and the FSHR. As shown in Table 1, all compounds from both series are potent full agonists for the LHR. Within series 1, only compound **IIC** and **IIF** show a two fold increase in potency compared to their monomeric counterparts **IC** and **IF**. This increase in potency is not observed on the FSHR. For all other dimeric compounds, the potencies are in the same range or decreased somewhat when compared to the monomeric analogs. On the FSHR, all compounds behave as partial agonists with an E_{max} of 52-82% compared to recombinant FSH. In addition, all dimeric ligands in this series show a reduced FSHR potency in comparison with the monomeric compounds. This decrease in FSH potency for the dimeric ligands generally results in more selective LHR agonists. For example, most dimers bearing a benzene ring in the spacer region are two to five times more selective for the LHR than their monomeric counterparts (**IA-F**). Exceptions are the dimeric ligands **IIIC**, derived from a valine spacer, and compounds **IIID** and **IVD** with amino butyric acid (Abu) spacer, both of which are less selective than their monomeric counterparts **IC** and **ID**.

For the compounds bearing flexible ethylene glycol spacers (series 2), a different pharmacological profile on the LHR and the FSHR is observed. Here, the monomeric compounds **4** and **12a-e** are more selective for the LHR than any of the compounds from series 1. The selectivity appears both dependent on the spacer system and the mode of attachment to ligand **3**. The monomeric compounds **12a-e** are more potent (~5-fold) on the LHR compared to the dimeric compounds **13a-e**. In contrast to the results from series 1, the potencies of the dimeric ligands **13a-e** on the FSHR are in the same order of magnitude as the monomeric compounds **12a-e**. Remarkably, the FSHR agonistic efficacy is significantly reduced upon dimerization (that is from **12a-e** to **13a-e**). Not more than 23% efficacy on the FSHR was observed at 10 μM concentrations of test compounds **13a-e** (Table 1 and Figure 4), while for all other compounds between 52-82% FSHR efficacy was observed at the highest test concentration (10 μM). In separate experiments it was established that none of the compounds from each series displays agonistic activity towards the TSHR at 10 μM concentration (data not shown).

The results obtained for the ligands in series 1 show some trends in agonistic potency on both LHR and FSHR. The spacer that is used for interconnecting the ligands has a significant influence on the activity of the compounds. For example, the monomeric compounds possessing a short, more rigid spacer (that is, alanine **IB**, valine **IC** and proline **IF**) are less potent on the LHR and the FSHR compared to the compounds with a more extended, flexible spacer such as those derived from glycine (**IA**), aminobutanoic acid (**ID**) and aminohexanoic acid (**IE**). This is also observed for some dimeric ligands in this series (for example, **IIIB** and **IIIC** compared to **IIIA** and **IIID** and **IIIE**). In most cases, the compounds with a spacer derived from aminobutyric acid (series **D**) provide the most potent LHR and FSHR agonists in this series (except **IIID** for LHR).
Some trends are also apparent for the compounds in series 2. A decrease in potency is observed for those compounds bearing an ethylene glycol moiety of increased length. This loss in activity may be due to the enhanced loss in entropy after ligand binding with increasing spacer length.

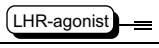
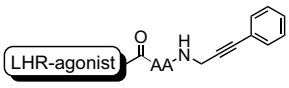
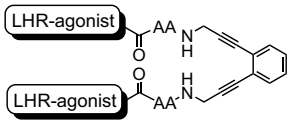
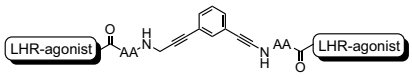
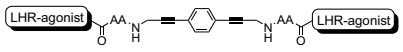
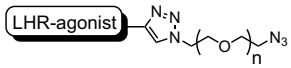
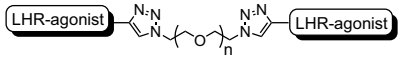
		Spacer (AA or n)	EC_{50} LHR (nM)	EC_{50} FSHR (nM)	E_{max}[a]	FSH/ LH[b]
	**3**	-	11	409	80 ± 4	39
	**4**	-	1	126	78 ± 9	137
	*Monomeric ligands **IA-F***					
	**IA**	Gly	8	243	72 ± 5	30
	IB	Ala	23	337	61 ± 4	16
	IC	Val	30	401	60 ± 2	13
	ID	Abu	5	124	52 ± 1	26
	IE	Ahx	9	275	54 ± 6	30
	IF	Pro	24	923	60 ± 1	39
	*Ortho-substituted dimeric ligands **IIA-F***					
	**IIA**	Gly	14	725	72 ± 3	51
	IIB	Ala	56	2240	62 ± 12	40
	IIC	Val	13	791	69 ± 3	62
	IID	Abu	12	483	74 ± 2	41
	IIE	Ahx	19	1010	82 ± 3	53
	IIF	Pro	14	1008	70 ± 4	72
	*Meta-substituted dimeric ligands **IIIA-F***					
	**IIIA**	Gly	51	2790	63 ± 2	55
	IIIB	Ala	285	>3000	n.d.	>11
	IIIC	Val	601	>3000	n.d.	>5
	IIID	Abu	26	264	61 ± 6	14
	IIIE	Ahx	24	2360	63 ± 8	99
	IIIF	Pro	37	2678	70 ± 8	72
	*Para-substituted dimeric ligands **IVA-F***					
	**IVA**	Gly	38	2030	61 ± 1	54
	IVB	Ala	142	>3000	n.d.	>21
	IVC	Val	110	>3000	n.d.	>27
	IVD	Abu	19	375	54 ± 0	20
	IVE	Ahx	38	2610	74 ± 4	68
	IVF	Pro	67	>3000	n.d.	>45
	*Monomeric ligands **12a-e***					
	**12a**	n = 0	4	548	71 ± 8	128
	12b	n = 1	5	504	75 ± 3	111
	12c	n = 2	5	397	81 ± 6	80
	12d	n = 3	7	595	80 ± 3	91
	12e	n = 4	6	706	72 ± 4	124
	*Dimeric ligands **13a-e***					
	**13a**	n = 0	16	780	23 ± 5	50
	13b	n = 1	19	494	17 ± 5	26
	13c	n = 2	23	527	21 ± 5	23
	13d	n = 3	25	475	19 ± 1	19
	13e	n = 4	28	506	18 ± 3	18

Table 1. Mean agonistic potency (EC_{50}) and selectivity for the LHR and FSHR. All compounds are full agonists for the LHR and partial agonists for the FSHR. The mean EC_{50} are calculated from the -log EC_{50} values from two or three independent experiments performed in duplicate. The SD of pEC_{50} is generally lower than 0.2. [a]. Maximal effect of the compounds on the FSHR. [b]. Selectivity for the LHR observed for the compounds (EC_{50} FSHR/EC_{50} LHR). n.d.: not determined. Abu: aminobutyric acid. Ahx: aminohexanoic acid.

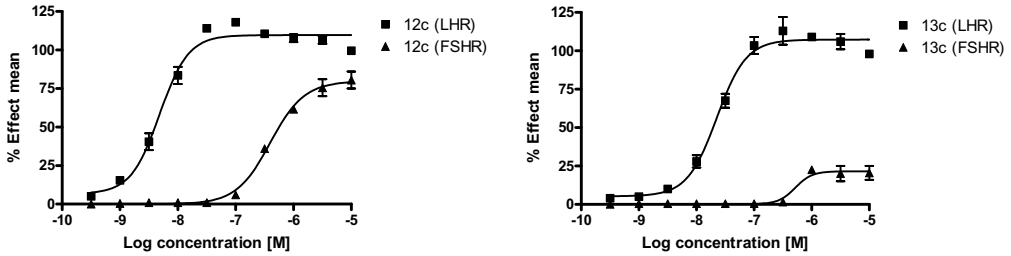


Figure 4. Representative example of the agonistic potencies of a selected monomer (**12c**, left) and dimer (**13c**, right) for the LHR and the FSHR bearing an ethylene glycol spacer. Effect is shown as percentage of the maximal luciferase activity induced by recombinant LH or FSH. Dimeric ligand **13c** exhibits a significant decrease in efficacy (E_{max} = 21%) on the FSHR compared to monomer **12c** (E_{max} = 81%).

The fact that most dimeric ligands show enhanced selectivity for the LHR compared to the corresponding monomers may result from preferential binding of both recognition units to one single LHR molecule in comparison to the FSHR. In order to evaluate whether the selectivity of the dimeric ligands stems from an intra-receptor interaction with a low-affinity binding site on the N-terminal LRR domain of the receptor, radioligand displacement studies with [^{125}I]-labeled hCG was performed. None of the ligands from series 1 induces more than 10% [^{125}I]-hCG displacement from the receptor at a concentration of 10 μM of test compound (data not shown). This result suggests that, in case a dimeric ligands bind to two distinct binding sites within the receptor, the second binding site is also located within the transmembrane (TM) region. Alternatively, there is some compelling literature evidence detailing that GpHRs exert their activity in dimerized form. Dimers of each of the three GpHRs have been observed to exist on the cell surface,[17-19] and in some examples for the LHR their occurrence appears to be agonist inducible.[20,21] Also, pairs of mutant GpH receptors either lacking the ligand binding domain or the G protein binding domain prove to function properly when coexpressed.[22-24] The latter suggests that receptor dimerization and/or cross-activation may provide a valid GpHR activation mechanism. Probably the most direct evidence of GpHR dimerization was obtained by the X-ray structure in which the FSHR ecto-domain was organized in a dimeric fashion.[25,26] These studies support the possibility that the dimeric ligands described here bind to two distinct receptors simultaneously. Despite the studies described above, structural evidence on the organization of the TM region of the GpH receptors remains rather limited, particularly in the presence of allosteric agonists such as described here. The selectivity observed for the dimeric ligands may therefore simply originate from the fact that the LHR is more tolerant towards modification of the parent ligand than the FSHR.

Conclusion

Disregarding which mode of binding outlined above is occurring, application of the bivalent ligand strategy to the family of GpH receptors has enabled the discovery of more selective LHR agonists compared to FSHR. Two series of dimeric ligands based on rigid, phenyl-substituted spacers and more flexible polyethylene glycol spacers were prepared. Either a decrease in potency

or a drop in efficacy on the FSHR caused the observed enhanced selectivity of the dimeric ligands compared to the corresponding monomeric counterparts. Based on these encouraging results, it would be interesting to further investigate the origin of the selectivity for GpH receptors in more detail. The fact that selectivity can be achieved for receptors with highly related TM regions may be used to further develop selective modulators on different (G-protein coupled) receptors.

Experimental procedures

Measurement of CRE-induced luciferase activity

Materials. Recombinant human LH (recLH) and human recombinant FSH (recFSH) were synthesized at Schering-Plough Research Institute, Oss, The Netherlands. Luclite® was obtained from Packard. All cell culture supplies were obtained from Gibco/BRL unless indicated otherwise. The human LH receptor cDNA[27] and human FSH receptor cDNA[28] were kindly provided by Dr. A.J.W. Hsueh, Stanford University.

Luciferase assay. Chinese Hamster Ovary (CHO)-K1 cells stably expressing the CRE-luciferase reporter with the human LH receptor or human FSH receptor were grown to 80-90% confluency in Dulbecco's MEM/Nutrient Mix F12 containing 5% bovine calf serum and supplemented with penicillin G (80 units/mL) and streptomycin (0.08 mg/mL) in 5% CO_2 at 37 °C. Cells were harvested using cell dissociation solution (Sigma). Aliquots of the cells were cryopreserved in DMSO without a loss of functional activity on LH receptor or FSH receptor.[29] On the day of the experiment, cells were thawed, washed with assay medium (Dulbecco's MEM/Nutrient Mix F12 supplemented with 1 mg/L bovine insulin (Sigma), 5 mg/L apo-transferrin (Sigma), penicillin G (80 units/mL) and streptomycin (0.08 mg/mL)) and then resuspended in assay medium. The compounds were tested at 10 concentrations ranging from final concentrations of 10 μM to 0.316 nM with half log intervals. In the agonistic assays, 10 μL of assay medium containing test compound and 3% DMSO, 10 μL of assay medium containing 3% DMSO with recLH (final concentration of 1 nM) or recFSH (final concentration of 586 pM) or 10 μL of assay medium containing 3% DMSO alone were added to the wells of a 384-well white culture plate followed by the addition of 10 μL of assay medium. Then, 10 μL of cell suspension containing 7,500 cells was added to the wells. The final concentration of DMSO was 1%. After incubation for 4 h in a humidified atmosphere in 5% CO_2 at 37 °C, plates were allowed to adjust to room temperature for 1 h. Then, 15 μL of LucLite solution (Packard) was added to the incubation mixture. Following 60 min at room temperature in the dark, luciferase activity was measured in a Packard Topcount Microplate Scintillation and Luminescence Counter. Agonistic effects of the compounds were determined as percentage of the (maximal) effect induced by 1 nM recLH or 586 pM recFSH. The EC_{50} values (concentration of the test compound that elicits half-maximal (50%) luciferase stimulation compared to the compound's maximally attainable effect, respectively) and the efficacy values (maximal effect as percentage of the effect of recLH or recFSH) of the test compounds were determined using the software program MathIQ (version 2.0, ID Business Solutions Limited).

Chemical procedures

Reactions were executed at ambient temperatures unless stated otherwise. All moisture sensitive reactions were performed under an argon atmosphere. All solvents were removed by evaporation under reduced pressure. Reactions were monitored by TLC analysis using silica gel coated plates (0.2 mm thickness) and detection by 254 nm UV-light or by either spraying with a solution of $(NH_4)_6Mo_7O_{24} \times 4H_2O$ (25 g/L) or $(NH_4)_4Ce(SO_4)_4 \times 2H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at ~150 °C. Column chromatography was performed on silica gel

(40-63 µm). NMR spectra were recorded on a 200/50 MHz, 300/75 MHz, 400/100 MHz, 500/125 MHz or 600/150 MHz spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants (*J*) are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. For LC-MS analysis, a HPLC-system (detection simultaneously at 214 and 254 nm) equipped with an analytical C_{18} column (4.6 mmD x 250 mmL, 5µ particle size) in combination with buffers A: H_2O, B: CH_3CN and C: 1% aq. TFA and coupled to a mass instrument with an electronspray interface (ESI) was used. For RP-HPLC purifications, an automated HPLC system equipped with a semi-preparative C_{18} column (5 µm C_{18}, 10Å, 150 x 21.2 mm) was used. The applied buffers were A: H_2O + ammonium acetate (20 mM) and B: CH_3CN. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60000 at *m/z* 400 (mass range *m/z* = 150-2000) and dioctylpthalate (*m/z* = 391.28428) as a lock mass. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan).

2-(3-(5-Amino-6-(tert-butylcarbamoyl)-2-(methylthio)thieno[2,3-d]pyrimidin-4yl)phenylamino) acetic acid (3). To a solution of amine **5**[9] (1.94 g, 5.0 mmol) in MeOH (50 mL) was added oxoacetic acid monohydrate (0.55 g, 6.0 mmol), acetic acid (0.73 mL, 10 mmol) and sodium acetate (0.41 g, 5.0 mmol). Subsequently sodium cyanoborohydride (0.33 g, 5.3 mmol) was added and the reaction mixture was stirred for 5 days. The mixture was evaporated and dissolved in ethyl acetate (100 mL) and water (50 mL). The organic layer was washed with brine (50 mL), dried (Na_2SO_4) and evaporated. The crude product was purified by silica gel column chromatography (0 to 10% methanol in dichloromethane) to yield 2.30 g of compound **3** (4.8 mmol, 95%) as a yellow solid. LC-MS analysis: t_R 9.73 min (linear gradient 10 to 90% B). ESI-MS *m/z*: 446.2 $[M + H]^+$. ^{1}H NMR (600 MHz, DMSO-*d*6) δ 7.29 (t, *J* = 7.2, 1H), 6.91 (s, 1H, N*H*), 6.80 (d, *J* = 6.6, 1H), 6.79 (s, 1H), 6.76 (d, *J* = 7.8, 1H), 6.10 (br s, 2H), 3.85 (s, 2H), 2.57 (s, 3H), 1.36 (s, 9H). ^{13}C NMR (150 MHz, DMSO-*d*6) δ 172.4 (C), 168.3 (C), 167.1 (C), 164.7 (CONH), 163.1 (C), 148.5 (C), 144.2 (C), 136.9 (C), 129.4 (CH), 117.3 (C), 116.0, 114.1, 111.4 (CH), 96.8 (C), 51.3 (C), 44.5 (CH_2), 28.7 (3 × CH_3), 13.8 (CH_3). HRMS *m/z*: calcd for $C_{20}H_{23}N_5O_3S_2$, + H^+: 446.13151, obsd 446.13149.

General procedure for coupling of pharmacophore 3 with monomeric spacers [0,1]-8A-F affording IA-F.

Boc-protected compounds **[0,1]-7A-F**[15] were subjected to a solution of 1/1 DCM/TFA v/v + 1% TIS for 18 h. The volatiles were evaporated and the compounds were purified with a semi-preparative HPLC system (0-40% B). Accordingly, 30 µmol of amine (**[0,1]-8A-F**) was dissolved in 100 µL of DMF and added to a solution containing pharmacophore **3** (30 µmol, 13.4 mg), BOP (39 µmol, 17.6 mg) and DiPEA (120 µmol, 20.4 µL) in 300 µL DMF. The reaction mixture was stirred at rt for 18h and diluted with a mixture of DCM and MeOH (9/1; v/v, 20 mL). The organic layer was successively washed with water (3 × 10 mL), 10% aqueous $NaHCO_3$ (3 × 10 mL) and brine (20 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated. The crude product was purified on a semi-preparative HPLC system (40 to 60% B) and lyophilized from dioxane/H_2O to obtain **1A-F** as yellow amorphous solids.

Monomeric ligand IA. Yield after RP-HPLC purification: 11.2 mg (15.2 µmol, 51%). LC-MS analysis: t_R 8.60 min (gradient 30 to 90% B). ESI-MS *m/z*: 616.1 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 – 7.30 (m, 6H), 7.18 (t, *J* = 4.2, 1H), 7.02 (d, *J* = 7.2, 1H), 6.83 (d, *J* = 8.2, 1H), 6.79 (s, 1H), 6.35 (t, *J* = 4.0, 1H), 5.98 (br s, 2H), 5.24 (s, 1H), 4.31 (d, *J* = 5.1, 2H), 4.03 (d, *J* = 5.2, 2H), 3.93 (s, 2H), 2.67 (s, 3H), 1.47 (s, 9H). HRMS *m/z*: calcd for $C_{31}H_{33}N_7O_3S_2 + H^+$: 616.21591, obsd 616.21584.

Monomeric ligand IB. Yield after RP-HPLC purification: 5.8 mg (7.7 µmol, 26%). LC-MS analysis: t_R 8.99 min (gradient 30 to 90% B). ESI-MS *m/z*: 630.1 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.30 (m, 6H), 7.07 (d, *J* = 7.6, 1H), 6.99 (d, *J* = 7.4, 1H), 6.79 (d, *J* = 6.8, 2H), 6.49 (t, *J* = 4.9, 1H), 6.23 – 5.84 (m, 2H), 5.24 (s, 1H), 4.60 – 4.51 (m, 1H), 4.31 – 4.23 (m, 2H), 3.89 (s, 2H), 2.66 (s, 3H), 1.48 (s, 9H), 1.42 (d, *J* = 6.9, 3H). HRMS *m/z*: calcd for $C_{32}H_{35}N_7O_3S_2 + H^+$: 630.23156, obsd 630.23148.

Monomeric ligand IC. Yield after RP-HPLC purification: 9.8 mg (12.6 µmol, 42%). LC-MS analysis: t_R 9.72 min (gradient 30 to 90% B). ESI-MS *m/z*: 658.1 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.27 (m, 7H), 7.11 (d, *J* = 8.8, 1H), 6.95 (d, *J* = 7.5, 1H), 6.77 (d, *J* = 9.5, 2H), 6.41 (t, *J* = 4.9, 1H), 5.94 (br s, 2H), 5.21 (s, 1H), 4.28 – 4.15 (m, 3H), 3.87 (s, 2H), 2.63 (s, 3H), 2.11 (m, *J* = 6.6, 1H), 1.46 (s, 9H), 0.90 (dd, *J* = 6.7, 25.5, 6H). HRMS *m/z*: calcd for $C_{34}H_{39}N_7O_3S_2 + H^+$: 658.26286, obsd 658.26280.

Monomeric ligand ID. Yield after RP-HPLC purification: 13.3 mg (17.4 µmol, 58%). LC-MS analysis: t_R 8.46 min (gradient 30 to 90% B). ESI-MS *m/z*: 644.2 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 – 7.32 (m, 6H), 6.98 (d, *J* = 8.1, 1H), 6.89 (s, 1H), 6.85 – 6.72 (m, 2H), 6.51 (s, 1H), 6.19 – 5.64 (m, 2H), 5.23 (s, 1H), 4.22 (d, *J* = 5.2, 2H), 3.87 (s, 2H), 3.39 (dd, *J* = 4.7, 7.6, 2H), 2.67 (s, 3H), 2.29 – 2.17 (m, 2H), 1.94 – 1.81 (m, 2H), 1.48 (s, 9H). HRMS *m/z*: calcd for $C_{33}H_{37}N_7O_3S_2 + H^+$: 644.24721, obsd 644.24710.

Monomeric ligand IE. Yield after RP-HPLC purification: 5.2 mg (6.5 µmol, 22%). LC-MS analysis: t_R 8.88 min (gradient 30 to 90% B). ESI-MS *m/z*: 672.3 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 – 7.28 (m, 6H), 6.97 (d, *J* = 7.4, 1H), 6.77 (d, *J* = 9.0, 2H), 6.60 (t, *J* = 6.2, 1H), 6.33 – 5.47 (m, 3H), 5.24 – 5.18 (m, 1H), 4.25 (d, *J* = 5.0, 2H), 3.82 (s, 2H), 3.29 (dd, *J* = 6.5, 12.7, 2H), 2.64 (s, 3H), 2.15 (t, *J* = 7.3, 2H), 1.69 – 1.48 (m, 4H), 1.45 (s, 9H), 1.33 – 1.22 (m, 2H). HRMS *m/z*: calcd for $C_{35}H_{41}N_7O_3S_2 + H^+$: 672.27851, obsd 672.27855.

Monomeric ligand IF. Yield after RP-HPLC purification: 9.1 mg (11.8 µmol, 46%). LC-MS analysis: t_R 9.39 min (gradient 30 to 90% B). ESI-MS *m/z*: 656.3 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 – 7.38 (m, 2H), 7.31 (dd, *J* = 7.1, 15.6, 4H), 7.17 (dd, *J* = 4.5, 9.6, 1H), 6.90 (d, *J* = 7.4, 1H), 6.81 (d, *J* = 8.2, 1H), 6.73 (s, 1H), 6.29 – 5.78 (m, 2H), 5.20 (s, 1H), 4.64 (d, *J* = 7.5, 1H), 4.25 (ddd, *J* = 5.3, 17.7, 22.3, 2H), 3.93 (s, 2H), 3.63 – 3.56 (m, 1H), 3.47 (dd, *J* = 8.4, 16.0, 1H), 2.64 (s, 3H), 2.50 – 2.42 (m, 1H), 2.30 – 2.15 (m, 1H), 2.11 – 2.00 (m, 1H), 1.98 – 1.84 (m, 1H), 1.44 (s, 9H). HRMS *m/z*: calcd for $C_{34}H_{37}N_7O_3S_2 + H^+$: 656.24721, obsd 656.24720.

General procedure for coupling of pharmacophore 3 with dimeric spacers [1,2]-10A-F, [1,3]-10AF or [1,4]-10A-F affording IIA-F, IIIA-F or IVA-F.

Boc protected compounds **[1,2]-9A-F**, **[1,3]-9A-F** or **[1,4]-9A-F**[15] were subjected to a solution of 1/1 DCM/TFA v/v + 1% TIS for 18 h. The volatiles were evaporated and the compounds were purified with a semi-preparative HPLC system (0 to 30% B). Accordingly, 15 µmol of amine (**[1,2]-10A-F**, **[1,3]-10A-F** or **[1,4]-10A-F**) was dissolved in 100 µL of DMF and added to a solution containing pharmacophore **3** (33 µmol, 14.8 mg), BOP (39 µmol, 17.6 mg) and DiPEA (120 µmol, 20.4 µL) in 300 µL DMF. The reaction mixture was stirred at rt for 18h and diluted with a mixture of DCM and MeOH (9/1; v/v, 20 mL). The organic layer was successively washed with

water (3 × 10 mL), 10% aqueous $NaHCO_3$ (3 × 10 mL) and brine (20 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated. The crude product was purified on a semi-preparative HPLC system (40 to 60% B) and lyophilized from dioxane/H_2O to obtain **IIA-F, IIIA-F or IVA-F** as yellow amorphous solids.

Dimeric ligand IIA. Yield after RP-HPLC purification: 5.1 mg (3.6 µmol, 24%). LC-MS analysis: t_R 10.9 min (gradient 30 to 90% B). ESI-MS *m/z*: 1153.4 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.66 (t, *J* = 4.8, 2H), 7.45 – 7.27 (m, 8H), 6.93 (d, *J* = 7.2, 2H), 6.75 (s, 4H), 6.33 – 5.60 (m, 4H), 5.25 (s, 2H), 4.26 (d, *J* = 5.1, 4H), 3.99 (d, *J* = 5.2, 4H), 3.90 (s, 4H), 2.65 (s, 7H), 1.46 (s, 18H). HRMS *m/z*: calcd for $C_{56}H_{60}N_{14}O_6S_4 + H^+$:1153.37758, obsd 1153.37702.

Dimeric ligand IIB. Yield after RP-HPLC purification: 6.5 mg (4.5 µmol, 31%). LC-MS analysis: t_R 10.7 min (gradient 30 to 90% B). ESI-MS *m/z*: 1181.4 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.48 (t, *J* = 5.3, 2H), 8.12 (d, *J* = 7.7, 2H), 7.38 (ddd, *J* = 3.5, 5.6, 46.7, 4H), 7.28 (t, *J* = 7.8, 2H), 6.90 (s, 2H), 6.81 – 6.73 (m, 6H), 6.29 (t, *J* = 5.8, 2H), 6.08 (br s, 4H), 4.39 – 4.33 (m, 2H), 4.20 – 4.11 (m, 4H), 3.82 – 3.72 (m, 4H), 2.57 (s, 6H), 1.35 (s, 18H), 1.21 (d, *J* = 7.0, 6H). HRMS *m/z*: calcd for $C_{58}H_{64}N_{14}O_6S_4 + H^+$: 1181.40888, obsd 1181.40848.

Dimeric ligand IIC. Yield after RP-HPLC purification: 4.2 mg (2.9 µmol, 19%). LC-MS analysis: t_R 12.6 min (gradient 30 to 90% B). ESI-MS *m/z*: 1237.5 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.56 (d, *J* = 8.5, 2H), 7.45 (t, *J* = 5.0, 2H), 7.40 – 7.33 (m, 4H), 7.27 – 7.20 (m, 6H), 6.92 (d, *J* = 7.6, 2H), 6.77 (s, 2H), 6.73 (d, *J* = 8.1, 2H), 5.25 (s, 2H), 4.46 (t, *J* = 6.0, 2H), 4.26 (ddd, *J* = 5.4, 17.7, 22.2, 4H), 3.88 (dd, *J* = 17.0, 23.8, 4H), 2.65 (s, 6H), 2.10 (ddd, *J* = 4.4, 9.6, 13.8, 2H), 1.47 (s, 18H), 0.91 (dd, *J* = 6.7, 27.2, 12H). HRMS *m/z*: calcd for $C_{62}H_{72}N_{14}O_6S_4 + H^+$: 1237.47149, obsd 1237.47125.

Dimeric ligand IID. Yield after RP-HPLC purification: 6.8 mg (4.7 µmol, 31%). LC-MS analysis: t_R 10.6 min (gradient 30 to 90% B). ESI-MS *m/z*: 1209.4 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.32 (m, 4H), 7.31 – 7.26 (m, 4H), 7.24 – 7.18 (m, 2H), 7.09 (t, *J* = 5.6, 2H), 6.92 (d, *J* = 7.3, 2H), 6.77 – 6.69 (m, 4H), 5.97 (br s, 4H), 5.26 (s, 2H), 4.23 (d, *J* = 5.3, 4H), 3.81 (s, 4H), 3.38 – 3.29 (m, 4H), 2.65 (s, 6H), 2.27 (t, *J* = 6.8, 4H), 1.86 (m, *J* = 6.0, 5H), 1.47 (s, 18H). HRMS *m/z*: calcd for $C_{60}H_{68}N_{14}O_6S_4 + H^+$: 1209.44019, obsd 1209.43993.

Dimeric ligand IIE. Yield after RP-HPLC purification: 2.5 mg (1.6 µmol, 11%). LC-MS analysis: t_R 11.1 min (gradient 30 to 90% B). ESI-MS *m/z*: 1265.5 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.32 (m, 4H), 7.24 (dd, *J* = 3.5, 5.6, 4H), 6.98 – 6.92 (m, 4H), 6.84 – 6.73 (m, *J* = 6.6, 15.2, 6H), 6.27 – 5.56 (m, 4H), 5.26 (s, 2H), 4.27 (d, *J* = 5.3, 4H), 3.80 (s, 4H), 3.26 (q, *J* = 6.6, 4H), 2.66 (s, 6H), 2.23 (t, *J* = 7.3, 4H), 1.72 – 1.56 (m, 4H), 1.47 (m, 22H), 1.36 – 1.24 (m, 4H). HRMS *m/z*: calcd for $C_{64}H_{76}N_{14}O_6S_4 + H^+$: 1265.50279, obsd 1265.50241.

Dimeric ligand IIF. Yield after RP-HPLC purification: 4.7 mg (3.2 µmol, 21%). LC-MS analysis: t_R 12.1 min (gradient 30 to 90% B). ESI-MS *m/z*: 1233.4 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.80 (s, 2H), 7.37 – 7.10 (m, 6H), 6.86 (d, *J* = 7.3, 2H), 6.80 (d, *J* = 8.2, 2H), 6.66 (s, 2H), 6.23 – 5.86 (br s, 4H), 5.20 (s, 2H), 4.51 (d, *J* = 6.5, 2H), 4.34 – 4.10 (m, 4H), 3.91 (s, 4H), 3.63 – 3.55 (m, 2H), 3.47 – 3.39 (m, 2H), 2.62 (s, 6H), 2.26 – 2.12 (m, 4H), 2.04 – 1.90 (m, 4H), 1.43 (s, 18H). HRMS *m/z*: calcd for $C_{62}H_{68}N_{14}O_6S_4 + H^+$: 1233.44019, obsd 1233.44059.

Dimeric ligand IIIA. Yield after RP-HPLC purification: 4.9 mg (3.5 µmol, 23%). LC-MS analysis: t_R 10.2 min (gradient 30 to 90% B). ESI-MS *m/z*: 1153.4 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.37 (m, 2H), 7.36 – 7.25 (m, 10H), 7.21 – 7.13 (m, 2H), 6.97 – 6.88 (m, 4H), 6.76 (d, *J* = 5.7, 4H), 6.19 – 5.69 (m, 4H), 5.26 (s, 2H), 4.23 (d, *J* = 4.8, 3H), 4.01 (d, *J* = 4.9, 4H), 3.85 (s, 4H), 2.65 (s, 6H), 1.46 (s, 18H). HRMS *m/z*: calcd for $C_{56}H_{60}N_{14}O_6S_4 + H^+$: 1153.37758, obsd 1153.37739.

Dimeric ligand IIIB. Yield after RP-HPLC purification: 5.5 mg (3.8 µmol, 26%). LC-MS analysis: t_R 10.7 min (gradient 30 to 90% B). ESI-MS *m/z*: 1181.4 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.35 – 7.13 (m, 12H), 6.99 (s, 2H), 6.95 (d, *J* = 7.4, 2H), 6.76 (d, *J* = 12.1, 4H), 6.18 – 5.81 (m, 4H), 5.26 (s, 2H), 4.64 – 4.55 (m, 2H), 4.22 (ddd, *J* = 5.2, 17.7, 22.3, 4H), 3.85 (s, 4H), 2.65 (s, 6H), 1.47 (s, 18H), 1.41 (d, *J* = 6.9, 6H). HRMS *m/z*: calcd for $C_{58}H_{64}N_{14}O_6S_4 + H^+$: 1181.40888, obsd 1181.40882.

Dimeric ligand IIIC. Yield after RP-HPLC purification: 3.3 mg (2.2 µmol, 15%). LC-MS analysis: t_R 11.7 min (gradient 30 to 90% B). ESI-MS *m/z*: 1237.5 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.35 – 7.12 (m, 12H), 7.01 (s, 2H), 6.94 (d, *J* = 7.3, 2H), 6.80 (s, 2H), 6.76 (d, *J* = 8.1, 2H), 6.19 – 5.76 (m, 4H), 5.26 (s, 2H), 4.41 – 4.35 (m, 2H), 4.23 (ddd, *J* = 5.1, 17.9, 22.2, 4H), 3.89 (s, 4H), 2.65 (s, 6H), 2.19 – 2.04 (m, 4H), 1.47 (s, 18H), 0.92 (dd, *J* = 6.7, 25.2, 12H). HRMS *m/z*: calcd for $C_{62}H_{72}N_{14}O_6S_4 + H^+$: 1237.47149, obsd 1237.47037.

Dimeric ligand IIID. Yield after RP-HPLC purification: 6.7 mg (4.6 µmol, 31%). LC-MS analysis: t_R 9.82 min (gradient 30 to 90% B). ESI-MS *m/z*: 1209.4 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.30 (dd, *J* = 8.0, 26.3, 10H), 7.21 – 7.16 (m, 2H), 6.98 (t, *J* = 5.6, 2H), 6.94 (d, *J* = 6.6, 2H), 6.78 (d, *J* = 8.1, 2H), 6.75 (s, 2H), 6.16 – 5.77 (m, 4H), 5.28 (s, 2H), 4.18 (d, *J* = 5.1, 4H), 3.83 (s, 4H), 3.36 (q, *J* = 5.4, 5H), 2.65 (s, 6H), 2.22 (t, *J* = 6.8, 4H), 1.91 – 1.82 (m, 4H), 1.47 (s, 18H). HRMS *m/z*: calcd for $C_{60}H_{68}N_{14}O_6S_4 + H^+$: 1209.44019, obsd 1209.44003.

Dimeric ligand IIIE. Yield after RP-HPLC purification: 4.6 mg (3.1 µmol, 20%). LC-MS analysis: t_R 10.5 min (gradient 30 to 90% B). ESI-MS *m/z*: 1265.5 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.40 – 7.17 (m, 12H), 6.97 (d, *J* = 6.9, 2H), 6.82 – 6.75 (m, 4H), 6.71 (t, *J* = 4.5, 2H), 6.37 (t, *J* = 6.0, 2H), 6.20 – 5.55 (m, 4H), 5.26 (s, 2H), 4.25 (d, *J* = 5.0, 4H), 3.83 (s, 4H), 3.30 (q, *J* = 4.8, 4H), 2.66 (s, 6H), 2.18 (t, *J* = 7.1, 4H), 1.73 – 1.56 (m, 8H), 1.48 (s, 18H), 1.34 – 1.22 (m, 4H). HRMS *m/z*: calcd for $C_{64}H_{76}N_{14}O_6S_4 + H^+$: 1265.50279, obsd 1265.50215.

Dimeric ligand IIIF. Yield after RP-HPLC purification: 4.5 mg (3.1 µmol, 21%). LC-MS analysis: t_R 11.3 min (gradient 30 to 90% B). ESI-MS *m/z*: 1233.4 $[M + H]^+$. ^1H NMR (400 MHz, CDCl3) δ 7.83 – 7.69 (m, 2H), 7.37 – 7.24 (m, 6H), 6.87 (d, *J* = 7.4, 2H), 6.83 – 6.74 (m, 2H), 6.72 (s, 1H), 6.04 (s, 4H), 5.22 (s, 2H), 5.04 (br s, 2H), 4.69 – 4.52 (m, 2H), 4.40 – 4.09 (m, 4H), 4.00 – 3.83 (m, 4H), 3.67 – 3.53 (m, 2H), 3.53 – 3.33 (m, 2H), 2.62 (s, 6H), 2.51 – 2.31 (m, 2H), 2.31 – 2.09 (m, 2H), 2.09 – 1.82 (m, 4H), 1.44 (s, 18H).HRMS *m/z*: calcd for $C_{62}H_{68}N_{14}O_6S_4 + H^+$: 1233.44019, obsd 1233.44059.

Dimeric ligand IVA. Yield after RP-HPLC purification: 5.8 mg (3.6 µmol, 24%). LC-MS analysis: t_R 10.0 min (gradient 30 to 90% B). ESI-MS *m/z*: 1153.4 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.40 (s, 2H), 7.35 – 7.26 (m, 2H), 7.21 (s, 4H), 6.98 – 6.86 (m, 4H), 6.76 (s, 4H), 6.17 – 5.80 (m, 4H), 5.27 (s, 2H), 4.24 (d, *J* = 3.2, 4H), 3.99 (d, *J* = 3.0, 4H), 3.88 (s, 4H), 2.64 (s, 6H), 1.46 (s, 18H). HRMS *m/z*: calcd for $C_{56}H_{60}N_{14}O_6S_4 + H^+$: 1153.37758, obsd 1153.37747.

Dimeric ligand IVB. Yield after RP-HPLC purification: 4.4 mg (3.1 µmol, 21%). LC-MS analysis: t_R 10.6 min (gradient 30 to 90% B). ESI-MS *m/z*: 1181.5 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.23 (m, 6H), 7.14 (d, *J* = 7.6, 2H), 6.97 (d, *J* = 7.3, 2H), 6.77 (d, *J* = 11.7, 4H), 6.20 – 5.75 (m, 4H), 5.26 (s, 2H), 4.68 – 4.49 (m, 2H), 4.25 (t, *J* = 4.7, 4H), 3.87 (s, 4H), 2.66 (s, 6H), 1.47 (s, 18H), 1.41 (d, *J* = 6.8, 6H). HRMS *m/z*: calcd for $C_{58}H_{64}N_{14}O_6S_4 + H^+$: 1181.40888, obsd 1181.40888.

Dimeric ligand IVC. Yield after RP-HPLC purification: 6.9 mg (4.7 µmol, 31%). LC-MS analysis: t_R 11.0 min (gradient 30 to 90% B). ESI-MS *m/z*: 1237.5 $[M + H]^+$. ^1H NMR (600 MHz, DMSO-*d*6) δ 8.57 (t, *J* = 5.4, 2H), 7.89 (d, *J* = 9.0, 2H), 7.35 (m, 4H), 7.28 (t, *J* = 7.8, 2H), 6.91 (s, 2H), 6.80 (dd, *J* = 1.3, 8.2, 4H), 6.73 (s, 2H), 6.37 (br s, 2H), 6.06 (br s, 4H), 4.19 (dd, *J* = 7.0, 8.7, 2H), 4.15 – 4.05 (m, 4H), 3.80 (dd, *J* = 16.5, 59.5, 4H), 2.57 (s, 6H), 1.92 (m, 2H), 1.35 (s, 18H), 0.76 (dd, *J* = 6.8, 22.7, 12H). HRMS *m/z*: calcd for $C_{62}H_{72}N_{14}O_6S_4 + H^+$: 1237.47149, obsd 1237.47100.

Dimeric ligand IVD. Yield after RP-HPLC purification: 5.3 mg (3.7 µmol, 24%). LC-MS analysis: t_R 9.63 min (gradient 30 to 90% B). ESI-MS *m/z*: 1209.4 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.39 – 7.17 (m, 6H), 6.96 (d, *J* = 7.7, 2H), 6.93 – 6.87 (m, 2H), 6.80 (d, *J* = 8.5, 2H), 6.76 (s, 2H), 6.69 – 6.64 (m, 2H), 6.13 – 5.99 (m, 4H), 5.26 (s, 2H), 4.21 (d, *J* = 4.8, 4H), 3.84 (s, 4H), 3.39 (q, *J* = 6.9, 4H), 2.66 (s, 6H), 2.24 (t, *J* = 6.4, 4H), 1.95 – 1.84 (m, 4H), 1.48 (s, 18H). HRMS *m/z*: calcd for $C_{60}H_{68}N_{14}O_6S_4 + H^+$: 1209.44019, obsd 1209.44042.

Dimeric ligand IVE. Yield after RP-HPLC purification: 3.1 mg (2.1 µmol, 14%). LC-MS analysis: t_R 10.3 min (gradient 30 to 90% B). ESI-MS *m/z*: 1265.5 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.36 (t, *J* = 7.7, 2H), 7.28 (s, 4H), 6.98 (d, *J* = 7.5, 2H), 6.79 (d, *J* = 10.9, 4H), 6.66 (t, *J* = 5.4, 2H), 6.14 (t, *J* = 4.6, 2H), 5.26 (s, 2H), 4.27 (d, *J* = 5.1, 4H), 3.84 (s, 4H), 3.31 (q, *J* = 6.3, 4H), 2.66 (s, 6H), 2.18 (t, *J* = 7.2, 5H), 1.69 – 1.60 (m, 4H), 1.54 – 1.42 (m, 22H), 1.34 – 1.23 (m, 4H). HRMS *m/z*: calcd for $C_{64}H_{76}N_{14}O_6S_4 + H^+$: 1265.50279, obsd 1265.50241.

Dimeric ligand IVF. Yield after RP-HPLC purification: 10.8 mg (5.6 µmol, 37%). LC-MS analysis: t_R 11.2 min (gradient 30 to 90% B). ESI-MS *m/z*: 1233.4 $[M + H]^+$. ^1H NMR (400 MHz, CDCl3) δ 7.38 – 7.25 (m, 6H), 6.89 (d, *J* = 7.1, 2H), 6.79 (d, *J* = 8.0, 2H), 6.72 (s, 2H), 6.05 (br s, 4H), 5.23 – 5.21 (m, 2H), 4.68 – 4.53 (m, 2H), 4.24 (ddd, *J* = 5.2, 17.6, 22.3, 4H), 4.03 – 3.85 (m, 4H), 3.64 – 3.54 (m, 2H), 3.54 – 3.36 (m, 2H), 2.65 (s, 6H), 2.51 – 2.38 (m, 2H), 2.33 – 2.11 (m, 2H), 2.10 – 1.99 (m, 2H), 1.99 – 1.84 (m, 2H), 1.45 (s, 18H). HRMS *m/z*: calcd for $C_{62}H_{68}N_{14}O_6S_4 + H^+$: 1233.44019, obsd 1233.43920.

5-Amino-*N*-*tert*-butyl-2-(methylthio)-4-(3-(2-oxo-2-(prop-2ynylamino)ethylamino)phenyl)thieno [2,3-*d*]pyrimidine-6-carboxamide (4). To a solution of **3**[9] (0.89 g, 2.0 mmol) and propargylamine (205 µL, 3.0 mmol) in DMF (20 ml) were added BOP (1.35 g, 3 mmol) and DiPEA (1.7 mL, 10.0 mmol) and the mixture was allowed to stir for 18 h. The solution was evaporated and the residue dissolved in EtOAc (200 mL) and washed with saturated aqueous $NaHCO_3$ (3 × 100 mL) and water (100 mL). The organic layer was dried ($MgSO_4$) and evaporated. The crude product was purified by silica gel column chromatography (0% to 50% EtOAc in toluene) to yield 0.83 g of **4** as a yellow solid (1.72 mmol, 86%). An analytical pure sample for biological evaluation was prepared by an additional purification on a semi-preparative RP-HPLC system (linear gradient of 3.0 CV; 65 to 80 %B). LC-MS analysis: t_R 9.16 min (linear gradient 10 to 90% B). ESIMS *m/z*: 483.2 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.33 (t, *J* = 7.8, 1H), 6.96 (d, *J* = 7.5, 1H), 6.86 (t, *J* = 5.1, 1H), 6.81 – 6.71 (m, 2H), 5.98 (s, 2H), 5.23 (s, 1H), 4.69 (s, 1H), 4.06 (dd, *J* = 1.9, 5.0, 2H), 3.82 (s, 2H), 2.63 (s, 3H), 2.19 (t, 1H), 1.44 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.8, 169.7, 167.3, 165.0, 162.6, 147.4, 144.6, 137.5 (8 × C), 130.0, 118.8 (2 × CH), 117.7 (C), 114.7, 113.3 (2 × CH), 96.7, 79.1 (2 × C), 71.7 (CH), 52.1 (C), 48.2, 29.1 (2 × CH_2), 28.9 (3 × CH_3), 14.4 (CH_3). HRMS *m/z*:

calcd for $C_{23}H_{26}N_6O_2S_2 + H^+$: 483.16314, obsd 483.16284.

General procedure for the preparation of monomeric ligands 12a-e.

A solution of the acetylene functionalized ligand **4** (90.2 mg, 0.15 mmol) and bis-azide spacer **11a, 11b, 11c, 11d** or **11e**[10] (1.5 mmol) in a mixture of *t*BuOH/CH_3CN/H_2O (2/2/1; v/v/v, 1 mL) was degassed for 1 h. Sodium ascorbate (5 eq. 375 µL of a 2 M solution in degassed H_2O) and $CuSO_4$ (1 eq. 75 µL of a 2 M solution in degassed H_2O) were added and the reaction mixture was stirred at 60 °C for 2h. The mixtures were diluted with MeOH/$CHCl_3$ (25 mL, 1/9) and washed with water (10 mL). The aqueous layer was extracted once with MeOH/$CHCl_3$ (25 mL, 1/9). The combined organic layers were dried ($MgSO_4$) and evaporated. The crude products were purified by silica gel column chromatography (0 to 20 % MeOH in EtOAc). An analytically pure sample for biological evaluation was prepared by additional purification on preparative RP-HPLC system (linear gradient of 3.0 CV; 65 to 80% B). Evaporation and lyophilization of the combined fractions furnished monovalent ligands **12a-e** as white amorphous powders.

Monomeric ligand 12a. Yield after RP-HPLC purification: 3.9 mg (5.5 µmol, 11%). LC-MS analysis: t_R 8.74 min (linear gradient 10 to 90% B). ESI-MS *m/z*: 595.27 $[M + H]^+$. ^1H NMR (400 MHz, DMSO-*d*6) δ 8.47 (t, *J* = 5.8, 1H), 7.88 (s, 1H), 7.30 (t, *J* = 7.9, 1H), 6.92 (s, 1H), 6.82 – 6.76 (m, 2H), 6.73 (s, 1H), 6.37 (t, *J* = 6.0, 1H), 6.10 (s, 2H), 4.49 (t, *J* = 5.4, 2H), 4.34 (d, *J* = 5.8, 2H), 3.78 (t, *J* = 5.8, 2H), 3.74 (d, *J* = 5.8, 2H), 2.58 (s, 3H), 1.35 (s, 9H). HRMS *m/z*: calcd for $C_{25}H_{30}N_{12}O_2S_2 + H^+$: 595.21289, obsd 595.21277.

Monomeric ligand 12b. Yield after RP-HPLC purification: 7.9 mg (10.4 µmol, 21%). LC-MS analysis: t_R 8.89 min (linear gradient 10 to 90% B). ESI-MS *m/z*: 639.20 $[M + H]^+$. ^1H NMR (400 MHz, DMSO-*d*6) δ 8.43 (t, *J* = 5.8, 1H), 7.80 (s, 1H), 7.30 (t, *J* = 7.8, 1H), 6.92 (s, 1H), 6.83 – 6.76 (m, 2H), 6.73 (s, 1H), 6.36 (t, *J* = 6.0, 1H), 6.11 (s, 2H), 4.48 (t, *J* = 5.3, 2H), 4.33 (d, *J* = 5.7, 2H), 3.80 (t, *J* = 5.3, 2H), 3.73 (d, *J* = 5.9, 2H), 3.54 (m, 2H), 3.36 – 3.33 (m, 2H), 2.58 (s, 3H), 1.36 (s, 9H). HRMS *m/z*: calcd for $C_{27}H_{34}N_{12}O_3S_2 + H^+$: 639.23910, obsd 639.23908.

Monomeric ligand 12c. Yield after RP-HPLC purification: 11.0 mg (13.7 µmol, 27%). LC-MS analysis: t_R 9.01 min (linear gradient 10 to 90% B). ESI-MS *m/z*: 683.27 $[M + H]^+$. ^1H NMR (400 MHz, DMSO-*d*6) δ 8.44 (t, *J* = 5.7, 1H), 7.81 (s, 1H), 7.30 (t, *J* = 7.8, 1H), 6.92 (s, 1H), 6.82 – 6.76 (m, 2H), 6.74 (s, 1H), 6.36 (t, *J* = 6.0, 1H), 6.11 (s, 2H), 4.46 (t, *J* = 5.2, 2H), 4.33 (d, *J* = 5.7, 2H), 3.77 (t, *J* = 5.3, 2H), 3.74 (d, *J* = 5.8, 2H), 3.55 – 3.52 (m, 2H), 3.51 (s, 4H), 3.37 – 3.33 (m, 2H), 2.58 (s, 3H), 1.36 (s, 9H). HRMS *m/z*: calcd for $C_{29}H_{38}N_{12}O_4S_2 + H^+$: 683.26532, obsd 683.26543.

Monomeric ligand 12d. Yield after RP-HPLC purification: 11.6 mg (13.7 µmol, 27%). LC-MS analysis: t_R 8.99 min (linear gradient 10 to 90% B). ESI-MS *m/z*: 727.33 $[M + H]^+$. ^1H NMR (400 MHz, DMSO-*d*6) δ 8.44 (t, *J* = 5.7, 1H), 7.81 (s, 1H), 7.30 (t, *J* = 7.8, 1H), 6.92 (s, 1H), 6.82 – 6.76 (m, 2H), 6.74 (s, 1H), 6.36 (t, *J* = 6.0, 1H), 6.11 (s, 2H), 4.45 (t, *J* = 5.3, 2H), 4.33 (d, *J* = 5.7, 2H), 3.77 (t, *J* = 5.4, 2H), 3.74 (d, *J* = 5.8, 2H), 3.59 – 3.55 (m, 2H), 3.54 – 3.46 (m, 8H), 3.38 – 3.34 (m, 2H), 2.58 (s, 3H), 1.36 (s, 9H). HRMS *m/z*: calcd for $C_{31}H_{42}N_{12}O_5S_2 + H^+$: 727.29153, obsd 727.29172.

Monomeric ligand 12e. Yield after RP-HPLC purification: 11.1 mg (12.5 µmol, 25%). LC-MS analysis: t_R 8.97 min (linear gradient 10 to 90% B). ESI-MS *m/z*: 771.33 $[M + H]^+$. ^1H NMR (400 MHz, DMSO-*d*6) δ 8.44 (t, *J* = 5.7, 1H), 7.81 (s, 1H), 7.30 (t, *J* = 7.8, 1H), 6.92 (s, 1H), 6.82 – 6.76 (m, 2H), 6.74 (s, 1H), 6.36 (t, *J* = 6.0, 1H), 6.11 (s, 2H), 4.45 (t, *J* = 5.3, 2H), 4.33 (d, *J* = 5.7, 2H), 3.76 (t, *J* = 5.4, 2H), 3.74 (d, *J* = 5.8, 2H), 3.60 – 3.57 (m, 2H), 3.55 – 3.44 (m, 12H), 3.39 – 3.35 (m, 2H), 2.58 (s, 3H), 1.36 (s, 9H). HRMS *m/z*: calcd for $C_{33}H_{46}N_{12}O_6S_2 + H^+$:

771.31775, obsd 771.31826.

General procedure for the preparation of dimeric ligands 13a-e.
A solution of the acetylene functionalized ligand **4** (24.0 mg, 40 μmol) and bis-azide spacer **11a, 11b, 11c, 11d** or **11e** (20 μmol) in a mixture of *t*BuOH/CH_3CN/H_2O (2/2/1; v/v/v, 800 μL) was degassed for 1h. Sodium ascorbate (5 eq. 200 μL of a 1 M solution in degassed H_2O) and $CuSO_4$ (1 eq. 40 μL of a 1 M solution in degassed H_2O) were added and the reaction mixture was stirred at 60 °C for 2h. The mixture was filtered and the crude products were purified by preparative RP-HPLC (linear gradient of 3.0 CV; 65 to 80% B). Evaporation and lyophilization of the combined fractions furnished dimeric ligands **13a-e** as white amorphous powders.

Dimeric ligand 13a. Yield after RP-HPLC purification: 11.1 mg (8.4 μmol, 42%). LC-MS analysis: t_R 10.45 min (linear gradient 10 to 90% B). ESI-MS *m/z*: 1077.6 $[M + H]^+$. ^1H NMR (400 MHz, DMSO-*d*6) δ 8.45 (t, *J* = 5.8, 2H), 7.72 (s, 2H), 7.29 (t, *J* = 7.7, 2H), 6.91 (s, 2H), 6.81 – 6.71 (m, 6H), 6.35 (t, *J* = 5.9, 2H), 6.10 (s, 4H), 4.79 (s, 4H), 4.29 (d, *J* = 5.7, 4H), 3.72 (d, *J* = 5.7, 4H), 2.57 (s, 6H), 1.35 (s, 18H). HRMS *m/z*: calcd for $C_{48}H_{56}N_{18}O_4S_4$ $+H^+$: 1077.36875, obsd 1077.36955.

Dimeric ligand 13b. Yield after RP-HPLC purification: 7.7 mg (5.7 μmol, 28%). LC-MS analysis: t_R 10.48 min (linear gradient 10 to 90% B). ESI-MS *m/z*: 1121.6 $[M + H]^+$. ^1H NMR (400 MHz, DMSO-*d*6) δ 8.43 (t, *J* = 5.7, 2H), 7.72 (s, 2H), 7.28 (t, *J* = 7.7, 2H), 6.91 (s, 2H), 6.82 – 6.72 (m, 6H), 6.35 (t, *J* = 5.9, 2H), 6.10 (s, 4H), 4.41 (t, *J* = 5.2, 4H), 4.32 (d, *J* = 5.5, 4H), 3.78 – 3.69 (m, 8H), 2.57 (s, 6H), 1.35 (s, 18H). HRMS *m/z*: calcd for $C_{50}H_{60}N_{18}O_5S_4$ $+H^+$: 1121.39497, obsd 1121.39576.

Dimeric ligand 13c. Yield after RP-HPLC purification: 10.7 mg (7.6 μmol, 38%). LC-MS analysis: t_R 10.37 min (linear gradient 10 to 90% B). ESI-MS *m/z*: 1165.5 $[M + H]^+$. ^1H NMR (400 MHz, DMSO-*d*6) δ 8.45 (t, *J* = 5.7, 2H), 7.79 (s, 2H), 7.29 (t, *J* = 7.8, 2H), 6.91 (s, 2H), 6.83 – 6.68 (m, 6H), 6.35 (t, *J* = 5.9, 2H), 6.11 (s, 4H), 4.43 (t, *J* = 5.2, 4H), 4.32 (d, *J* = 5.5, 4H), 3.78 – 3.66 (m, 8H), 3.44 (s, 4H), 2.58 (s, 6H), 1.35 (s, 18H). HRMS *m/z*: calcd for $C_{52}H_{64}N_{18}O_6S_4$ $+H^+$: 1165.42118, obsd 1165.42228.

Dimeric ligand 13d. Yield after RP-HPLC purification: 14.5 mg (10.0 μmol, 50%). LC-MS analysis: t_R 10.34 min (linear gradient 10 to 90% B). ESI-MS *m/z*: 1209.5 $[M + H]^+$. ^1H NMR (400 MHz, DMSO-*d*6) δ 8.43 (t, *J* = 5.7, 2H), 7.80 (s, 2H), 7.29 (t, *J* = 7.8, 2H), 6.90 (s, 2H), 6.82 – 6.69 (m, 6H), 6.35 (t, *J* = 6.0, 2H), 6.10 (s, 4H), 4.44 (t, *J* = 5.2, 4H), 4.32 (d, *J* = 5.5, 4H), 3.74 (t, *J* = 5.5, 8H), 3.51 – 3.44 (m, 4H), 3.44 – 3.39 (m, 4H), 2.58 (s, 6H), 1.35 (s, 18H). HRMS *m/z*: calcd for $C_{54}H_{68}N_{18}O_7S_4$ $+H^+$: 1209.44740, obsd 1209.44822.

Dimeric ligand 13e. Yield after RP-HPLC purification: 13.4 mg (9.0 μmol, 45%). LC-MS analysis: t_R 10.28 min (linear gradient 10 to 90% B). ESI-MS *m/z*: 1253.5 $[M + H]^+$. ^1H NMR (400 MHz, DMSO-*d*6) δ 8.43 (t, *J* = 5.7, 2H), 7.80 (s, 2H), 7.29 (t, *J* = 7.8, 2H), 6.90 (s, 2H), 6.82 – 6.71 (m, 6H), 6.35 (t, *J* = 5.9, 2H), 6.10 (s, 4H), 4.44 (t, *J* = 5.2, 4H), 4.33 (d, *J* = 5.7, 4H), 3.81 – 3.70 (m, 8H), 3.50 – 3.40 (m, 12H), 2.58 (s, 6H), 1.35 (s, 18H). HRMS *m/z*: calcd for $C_{56}H_{72}N_{18}O_8S_4$ $+H^+$: 1253.47361, obsd 1253.47465.

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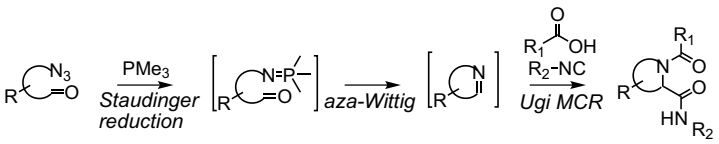
Chapter 6

Hydroxylated prolines as spacers for dimeric LHR agonists

Introduction

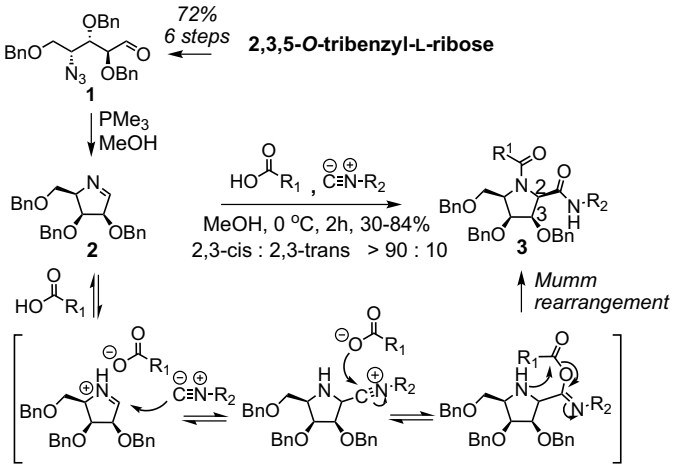
Multicomponent reactions (MCRs) are frequently used in organic and medicinal chemistry research as a powerful method to generate large families of structurally related molecules. Among MCRs, the Ugi reaction is one of the most explored to date.[1-4] In the Ugi four-component reaction (Ugi-4CR), an aldehyde, an amine, a carboxylic acid and an isocyanide, all of which may possess a variety of different functionalities, are combined to form a bisamide.The first step in this process is the condensation of the aldehyde and amine to an intermediate imine, which reacts with the carboxylic acid and isocyanide entities to give the Ugi product. In the Ugi-three component reaction (Ugi-3CR), a preformed imine is mixed with a carboxylic acid and an isocyanide to give a bisamide.

A variation on the Ugi reaction is called the tandem Staudinger/aza-Wittig/Ugi-three component reaction (SAWU-3CR, Scheme 1).[5] In this process an azido aldehyde[6] is reacted with a trialkylphosphine (Staudinger reaction) to give an intermediate phosphazene, which undergoes an intramolecular aza-Wittig reaction with the aldehyde moiety to provide a cyclic imine. Addition of an isocyanide and a carboxylic acid at this stage provides a bisamide in an Ugi-3CR sequence of events.



Scheme 1. Overview of the SAWU-3CR reaction sequence.

The SAWU-3CR was previously employed to transform carbohydrate derived azido-aldehyde **1** into highly functionalized pyrrolidines (**3**, Scheme 2).[7,8] The obtained pyrrolidine motif[9-13] can be seen as a carbohydrate in which the ring oxygen is replaced by a nitrogen atom, often referred to as an iminosugar, or as a hydroxylated proline analogue. Iminosugars are efficient glycosidase or glycosyltransferase inhibitors[14,15] and hydroxylated prolines can have a great influence on polypeptide secondary structures and (bio)physical properties.[16-18]



Scheme 2. Synthesis of pyrrolidines from azido-aldehyde **1** with the tandem SAWU-3CR.

In chapter 5 the synthesis and biological evaluation of dimeric luteinizing hormone receptor (LHR) agonists are described. The linker systems for interconnecting the two pharmacophores were based on a rigid benzene substituted core or on a more flexible ethylene-glycol system. Biological results of these compounds revealed that the dimeric ligands have improved selectivity for the LHR when compared to the monomeric ligands that also affect the follicle-stimulating hormone receptor (FSHR).[19-22] Within the series based on rigid benzene substituted linkers, a clear trend was observed that the agonistic LHR and FSHR potencies were lower for compounds with more rigid and lipophilic spacers (for example, with alanine, valine and to a lesser extend with proline) than for compounds with more flexible spacers. To elucidate whether this phenomenon may be ascribed to lipophilicity rather than rigidity of the linker system, a set of compounds was designed that contain hydroxylated prolines in the spacer system generated by the SAWU-3CR (Figure 1). The hydroxylated proline derivatives are designed such that the linkers between the LHR agonists are of the same length and orientation as the proline-containing compounds described in Chapter 5 (exemplified in Figure 1).

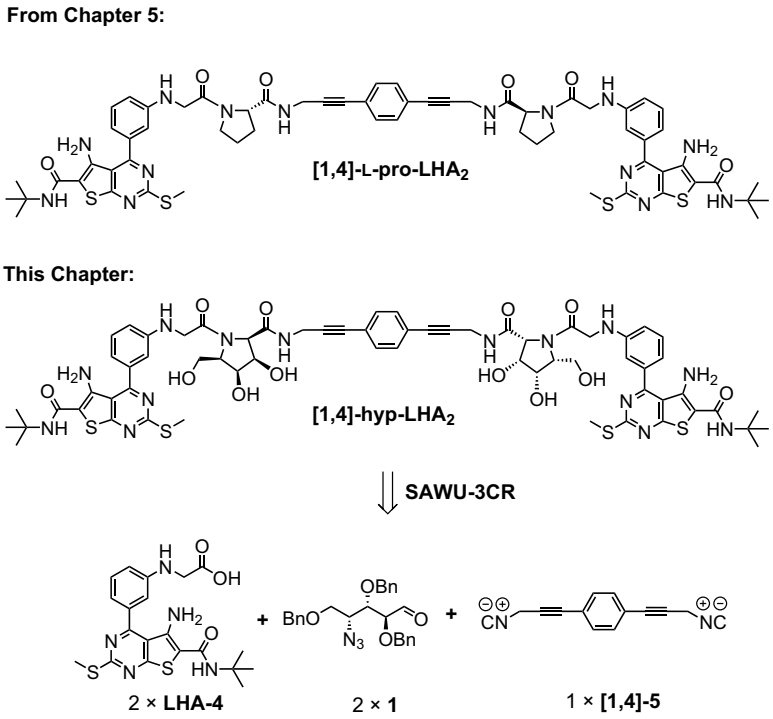
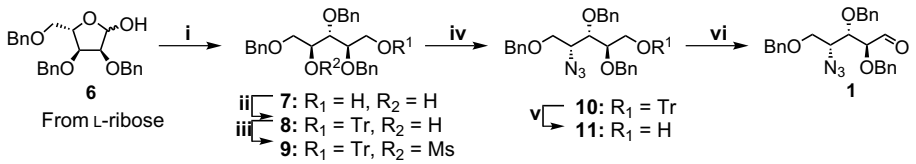


Figure 1. General strategy for the preparation of dimeric ligand bearing hydroxylated proline spacers with the SAWU-3CR.

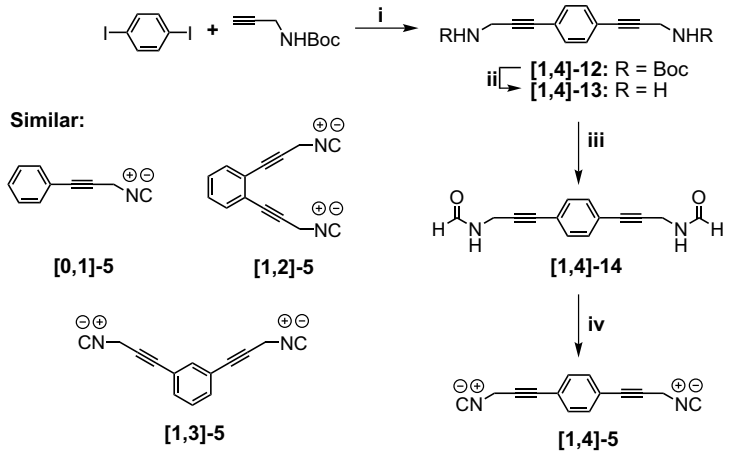
Results and Discussion

The requisite building blocks for the SAWU-3CR to obtain hydroxylated proline (hyp) ligand **[1,4]-hyp-LHA$_2$** are the LH agonist **LHA-4**[23] bearing a carboxylic acid functionality, the 4-azidopentanal **1** and bis-isocyanide **[1,4]-5** (Figure 1). The 4-azidopentanal **1** was synthesized from L-ribose (Scheme 3) as follows. The hemiacetal function in 2,3,5-tri-*O*-benzyl-L-ribofuranose (**6**)[24] was reduced with sodium borohydride and the resulting free primary hydroxyl function of **7** was selectively protected as trityl ether **8**. Subsequent mesylation of the remaining hydroxyl function provided L-ribitol derivative **9**. Exposing the mesylate in **9** to excess sodium azide at 90 °C in DMF in the presence of 20 mol% of 15-crown-5 ether and tetrabutylammonium hydrogen sulfate[25] furnished azide **10** in excellent yield. Removal of the trityl group under acidic conditions and ensuing oxidation of alcohol **11** with Dess-Martin periodinane provided 4-azidopentanal **1** in 72% yield over the six steps.



Scheme 3. Synthesis of 4-azidopentanal **1**. *Reagents and conditions*: *i.* $NaBH_4$, EtOH, 0 °C, 3 h; *ii.* TrCl, Et_3N, DCM, 16 h, 89% over two steps; *iii.* MsCl, pyridine, 5 °C, 16 h, 98%; *iv*. 6 eq NaN_3, 20 mol% 15-crown-5, 20 mol% Bu_4NHSO_4, 90 °C, 2 days, 94%; *v*. cat. *p*-TsOH, $CHCl_3$/MeOH; 1/1, 16 h, 88%; *vi.* Dess-Martin periodinane, DCM, 0 °C, 3 h, quant.

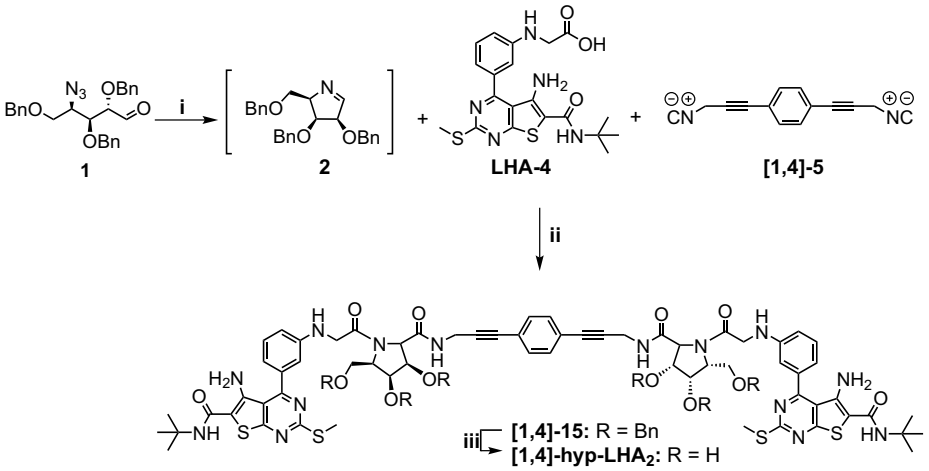
The synthesis of bis-isocyanide **[1,4]-5** is depicted in Scheme 4. Boc-protected propargyl amine was subjected to 1,4-diiodobenzene in a Sonogashira coupling reaction with CuI and $Pd(PPh_3)_4$ in a pyrrolidine/DMF mixture to obtain compound **[1,4]-12** in 91% yield. Removal of the Boc protective group (TFA/DCM) and subsequent treatment of the crude bis-amine **[1,4]-13** with ethyl formate and triethyl amine in refluxing ethanol provided formamide **[1,4]-14** in 76% yield over the two steps. Dehydration of the formamide with $POCl_3$ in DCM provided the bis-isocyanide **[1,4]-5** in quantitative yield. Following this route, three bis-isocyanides were prepared, having a different substitution pattern on the phenyl ring (that is, *ortho*, *meta* and *para*), as well as one monomeric reference compound (**[0,1]-5**).



Scheme 4. Synthesis of (bis)-isocyanides **5**. *Reagents and conditions*: *i*. $Pd(PPh_3)_4$, CuI, pyrrolidine, DMF, 18 h, 91%; *ii*. 20% TFA/DCM, 18 h; *iii*. Ethylformate, EtOH, Et_3N, reflux, 18h. 76% over two steps. *iv*. $POCl_3$, Et_3N, DCM, -20 °C, 40-99%.

The construction of the dimeric ligands with the SAWU-3CR is depicted in Scheme 5. 4-Azidopentanal **1** was subjected to a SAWU-3CR process by reaction with trimethyl phosphine in methanol at 0 °C, until TLC analysis showed complete consumption of **1**. The reaction mixture was concentrated and remaining solvents were removed by coevaporation with toluene to give pyrroline **2** as the crude intermediate cyclic imine. A methanolic solution of **2** at 0 °C was charged with 1.2 equivalents of LH agonist (**LHA-4**) and 0.4 equivalents of bis-isocyanide **[1,4]-5** and stirred for 16 hours at room temperature. Concentration and purification by size exclusion and silica gel column chromatography provided the SAWU-3CR product **[1,4]-15** in 32% yield (Scheme 5). Cleavage of the benzyl protective groups with classical hydrogenation conditions (Pd/C, H_2) was expected to be difficult due to the lability of the propargyl functionalities in the linker system under these conditions. Indeed, several hydrogenation experiments did not result in the adequate formation of the final compounds. The benzyl groups could however be cleaved with reasonable conversion with BCl_3 in DCM as judged by LCMS analysis. The target compound was subsequently purified by preparative HPLC to afford compound **[1,4]-hyp-LHA$_2$** in 10% yield. In this fashion three dimeric compounds **[1,2]-hyp-LHA$_2$**, **[1,3]-hyp-LHA$_2$** and **[1,4]-hyp-LHA$_2$** and one monomeric compound **[0,1]-hyp-LHA** were prepared (the structures of these are depicted in Figure 2).

In previous studies on the scope of the SAWU-3CR, it was observed that azido-aldehyde **1** produced pyrrolidines with counter-intuitive 2,3-*cis* stereochemistry at the new stereogenic center (Scheme 2) as was determined with NOESY NMR experiments.[7] The final Ugi-3CR step with the intermediate cyclic imine **2** establishes this stereochemistry (see the mechanism of formation, Scheme 2) and appears not to be dependent on the used carboxylic acid- or isocyanide component.



Scheme 5. Synthesis of dimeric ligands bearing oligohydroxy-substituted proline spacers via the SAWU-3CR. *Reagents and conditions*: *i*. PMe_3, MeOH, 0 °C, 2h; *ii*. MeOH, 0 °C, 16 h, 32% overall yield; *iii*. BCl_3, DCM, 0 °C to rt, preparative HPLC.

For monomeric ligand **[0,1]-hyp-LHA** it could be established by NOESY NMR that the newly formed stereocenter was also in the 2,3-*cis* fashion. For the dimeric ligands it was not possible to determine the exact configuration of the newly formed stereo-centers due to peak-broadening, the presence of rotamers and overlap of signals in ^{1}H NMR. However, based on the mechanism of the Ugi-reaction and the fact that formation of the 2,3-*trans* pyrrolidines was not observed previously, it is assumed that both newly formed stereocenters are in a 2,3-*cis* relationship.

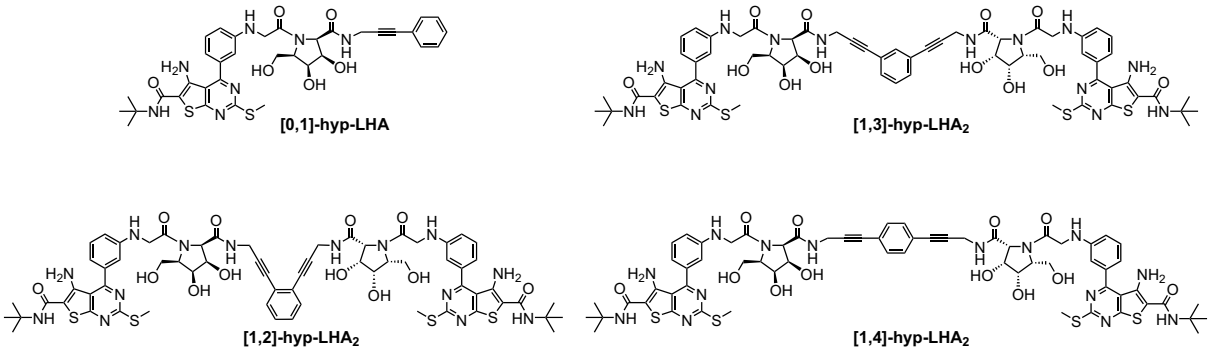
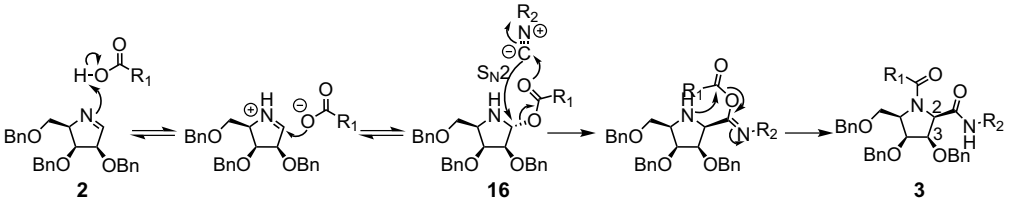


Figure 2. Structures of the prepared monomeric **[0,1]-hyp-LHA** and dimeric compounds **[1,2]-hyp-LHA$_2$**, **[1,3]-hyp-LHA$_3$** and **[1,4]-hyp-LHA$_2$**, via the SAWU-3CR.

A possible explanation for the diastereoselectivity in 2,3-*cis* pyrrolidine formation (**3**) in the Ugi-3C reaction with D-*lyxo*-pyrroline **2** may be found in the existence of an acyloxy intermediate in the course of the reaction (furnishing acyloxy **16**, Scheme 6). Acyloxy intermediates were first postulated by Ugi *et al* and their involvement in certain Ugi reactions has subsequently been proposed by others.[26-28,4,12] Intermediate **16** would be formed after protonation of the imine by attack of the nearby carboxylate anion from the least hindered side. After S_N2 displacement by the isocyanide and subsequent Mumm-rearrangement this pathway would lead to 2,3-*cis* pyrrolidines **3**. Alternatively, the carboxylate might also form a contact ion pair with the protonated cyclic imine and thereby shield one face from isocyanide attack.



Scheme 6. Proposed mechanism for the formation of 2,3-*cis* pyrrolidines **3** via acyloxy intermediate **16**.

Another plausible explanation for the 2,3-*cis* pyrrolidine **3** formation involves the influence of electronic effects on the conformation of cyclic imine **2**. Woerpel and co-workers proposed a model for nucleophilic additions to five-membered ring oxocarbenium ion electrophiles.[29,30] In this model, a pseudoaxial position of alkoxy substituents at C-3 produces the lowest energy conformer of the oxocarbenium ion by maximizing favorable intramolecular electrostatic interactions (note that C-3 in oxocarbenium ions corresponds to C-4 in pyrroline **2**, that for clarity reasons is numbered similarly to pyrrolidines **3**). The nucleophile then attacks from the concave site of the envelope conformation giving a 1,3-*cis* product. Similarly, D-*lyxo*-pyrroline **2** may adopt an envelope conformation in which the C-3/C-4/C-5 substituents also possess the required stereochemistry for the favored pseudoaxial orientation of C-4 that promotes 2,4-*cis* attack. In the Ugi-3CR with **2** this would give 2,3-*cis* products (Figure 3).

Woerpel and co-workers further demonstrated the 'critical' electronic effect of the pseudoaxial C3 alkoxy by testing a C-3 inverted D-arabinose analogue.[30] The C-3 pseudoaxial conformer for this oxocarbenium ion is disfavored and a nucleophilic addition reaction with it produced a 50:50 mixture of 1,3-*cis* and 1,3-*trans* attack products. Analogously, the Ugi-3CR on D-*arabino*-pyrroline **17** gave a 54:46 2,3-*cis*/*trans* product ratio as opposed to the >90:10 mixture obtained for D-*lyxo*-pyrroline **2**. The loss of selectivity observed for **17** is in agreement with the model presented in Figure 3.

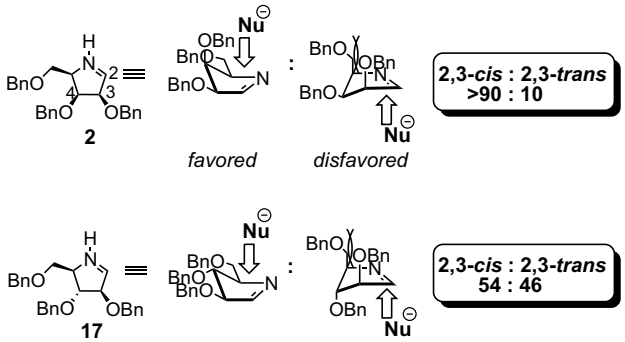


Figure 3. Electrostatic stabilization of iminium intermediate **2** by 4-alkoxy substituents.

Chapter 5 describes the synthesis of a set of monomeric and dimeric ligands that contain a L-proline moiety in the spacer system. Since the newly formed stereocenter in the monomeric and dimeric ligands formed by the SAWU-3CR corresponds to the D-proline conformation, the reference compounds bearing a D-proline in the spacer moiety were prepared as described in chapter 5.

All compounds were tested on agonistic activity on the LHR and the FSHR (listed in Table 1). All compounds are full agonists for the LHR and partial agonists for the FSHR. Some remarkable activities are observed for the compounds bearing a proline in the linker. For example, the monomeric compound **[0,1]-D-pro-LHA** is slightly less potent on the LHR than **[0,1]-L-pro-LHA** while the potencies on the FSHR are slightly higher. This suggests that the nature of the linker, because of the opposing stereochemical properties, partly governs the observed potencies on both of the receptors. The potencies of dimeric compounds on the LHR are in the same order of magnitude for the molecules with either a D-proline or a L-proline in the linker. Only the *ortho*-substituted compound **[1,2]-L-pro-LHA$_2$** shows reasonable agonistic potency on the FSHR while the other dimeric compounds are significantly less potent ($P < 0.05$).
While the monomeric compound **[0,1]-hyp-LHA** shows potencies on both the LHR and the FSHR that are in the same order of magnitude as the compounds bearing a proline in the linker, the dimeric hyp-ligands differ remarkably in their activity profile. Here, *ortho*-substituted dimer **[1,2]-hyp-LHA$_2$** and *para*-substituted dimer **[1,4]-hyp-LHA$_2$** exhibit more than 10 times lower LHR potency compared to the *meta*-substituted dimer **[1,3]-hyp-LHA$_2$**. Moreover, the *meta*-dimer **[1,3]-hyp-LHA$_2$** is a significant more potent LHR agonist and a less potent FSH agonist than the corresponding monomeric compound **[0,1]-hyp-LHA** ($P<0.05$). It is reasonable to assume that the compounds bearing a rigid benzene substituted core, apart from receptor binding, also have a strong interaction with the cell-membrane. The here described compounds are more hydrophilic due to the multiple hydroxyl functions and may therefore show a different receptor binding and kinetic profile. The *meta*-substituted compound **[1,3]-hyp-LHA$_2$** seems to have a more favorable orientation to interact with the receptor than the *ortho*- and *para*-dimers. From the results in Table 1, it may be concluded that the geometrical constraints for optimal LHR binding are more pronounced in the hyp-LHA$_2$ series compared to the pro-LHA$_2$ series. The spatial orientation, imposed by the substitution pattern of the linkers on the benzene core system

seems to contribute less to the potency for the more lipophilic proline-series. When more hydrogen-bond forming potential is introduced in the linker system, the substitution pattern becomes a potency-determining factor.

From Chapter 5 it is evident that dimeric ligands may possess an increase in selectivity for the LHR over the FSHR compared to the monomeric compounds. From the results presented in Table 1, it can be concluded that this is a general trend for the dimeric ligands that it is not related to the nature of the linker part.

	EC_{50} (nM)			
Compound	**LHR**	**FSHR**	**E_{max} (%)[a]**	**Selectivity[b]**
[0,1]-L-pro-LHA	24	923	60	39
[1,2]-L-pro-LHA_2	14	1008	70	72
[1,3]-L-pro-LHA_2	37	2678	70	72
[1,4]-L-pro-LHA_2	67	>3000	33 % at 3160 nM	>45
[0,1]-D-pro-LHA	33	831	46	25
[1,2]-D-pro-LHA_2	20	2941	63	151
[1,3]-D-pro-LHA_2	28	>3000	29 % at 3160 nM	>107
[1,4]-D-pro-LHA_2	25	>3000	29 % at 3160 nM	>122
[0,1]-hyp-LHA	27	749	60	28
[1,2]-hyp-LHA_2	244	>3000	6 % at 3160 nM	>12
[1,3]-hyp-LHA_2	14	1408	71	98
[1,4]-hyp-LHA_2	119	>3000	14 % at 3160 nM	>25

Table 1. Agonistic potencies for the compounds on the LHR and FSHR. All compounds are full agonists for the LHR and partial agonists for the FSHR. EC_{50} are determined from two or three independent experiments performed in duplicate. The SD of pEC_{50} is generally lower than 0.2. a. Maximal effect observed for monomeric and dimeric compounds on the FSHR. For compounds with an EC_{50} >3000 nM on the FSHR, the % effect at 3160 nM is reported. b. Selectivity observed for the LHR (EC_{50} FSHR/EC_{50} LHR).

Conclusion

In conclusion, the SAWU-3CR was applied in the preparation of monomeric and dimeric ligands that agonize the LHR. The resulting molecules bear a proline linker system that was decorated with multiple hydroxyl functions. The newly formed stereo-centers appear to be in a 2,3-*cis* relation, which is in agreement with previously reported stereogenic outcome using D-*lyxo*-pyrroline **2** as in the SAWU-3CR. The monomeric compound prepared in this fashion possesses similar LHR and FSHR agonistic activities as was observed with the compounds that lack the hydroxyl functions. Remarkably, the *ortho*-and *para*-substituted compounds with the hydroxylated proline spacer show a reduced activity (10- and 5-fold) for the LHR while the *meta*-dimer was significantly more active than the corresponding monomer. In all cases, the dimeric compounds were more selective for the LHR than the monomeric compounds. This is in agreement with the results from the studies described in Chapter 5.

Experimental section

Measurement of CRE-induced luciferase activity

Materials. Recombinant human LH (recLH) and human recombinant FSH (recFSH) were synthesized at Schering-Plough Research Institute, Oss, The Netherlands. Luclite® was obtained from Packard. All cell culture supplies were obtained from Gibco/BRL unless indicated otherwise. The human LH receptor cDNA[31] and human FSH receptor cDNA[32] were kindly provided by Dr. A.J.W. Hsueh, Stanford University.

Luciferase assay. Chinese Hamster Ovary (CHO)-K1 cells stably expressing the CRE-luciferase reporter with the human LH receptor or human FSH receptor were grown to 80-90% confluency in Dulbecco's MEM/Nutrient Mix F12 containing 5% bovine calf serum and supplemented with penicillin G (80 units/mL) and streptomycin (0.08 mg/mL) in 5% CO_2 at 37 °C. Cells were harvested using cell dissociation solution (Sigma). Aliquots of the cells were cryopreserved in DMSO without a loss of functional activity on LH receptor or FSH receptor.[33] On the day of the experiment, cells were thawed, washed with assay medium (Dulbecco's MEM/Nutrient Mix F12 supplemented with 1 mg/L bovine insulin (Sigma), 5 mg/L apo-transferrin (Sigma), penicillin G (80 units/mL) and streptomycin (0.08 mg/mL)) and then resuspended in assay medium. The compounds were tested at 10 concentrations ranging from final concentrations of 10 μM to 0.316 nM with half log intervals. In the agonistic assays, 10 μL of assay medium containing test compound and 3% DMSO, 10 μL of assay medium containing 3% DMSO with recLH (final concentration of 1 nM) or recFSH (final concentration of 586 pM) or 10 μL of assay medium containing 3% DMSO alone were added to the wells of a 384-well white culture plate followed by the addition of 10 μL of assay medium. Then, 10 μL of cell suspension containing 7,500 cells was added to the wells. The final concentration of DMSO was 1%. After incubation for 4 h in a humidified atmosphere in 5% CO_2 at 37 °C, plates were allowed to adjust to room temperature for 1 h. Then, 15 μL of LucLite solution (Packard) was added to the incubation mixture. Following 60 min at room temperature in the dark, luciferase activity was measured in a Packard Topcount Microplate Scintillation and Luminescence Counter. Agonistic effects of the compounds were determined as percentage of the (maximal) effect induced by 1 nM recLH or 586 pM recFSH. The EC_{50} values (concentration of the test compound that elicits half-maximal (50%) luciferase stimulation compared to the compound's maximally attainable effect, respectively) and the efficacy values (maximal effect as percentage of the effect of recLH or recFSH) of the test compounds were determined using the software program MathIQ (version 2.0, ID Business Solutions Limited).

Chemical procedures

Reactions were executed at ambient temperatures unless stated otherwise. All moisture sensitive reactions were performed under an argon atmosphere. All solvents were removed by evaporation under reduced pressure. Reactions were monitored by TLC analysis using silica gel coated plates (0.2 mm thickness) and detection by 254 nm UV-light or by either spraying with a solution of $(NH_4)_6Mo_7O_{24} \times 4H_2O$ (25 g/L) or $(NH_4)_4Ce(SO_4)_4 \times 2H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at ~150 °C. Column chromatography was performed on silica gel (40-63 μm). NMR spectra were recorded on a 200/50 MHz, 300/75 MHz, 400/100 MHz, 500/125 MHz or 600/150 MHz spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants (*J*) are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. Where indicated, NMR peak assignments were made using COSY, NOESY (τ mix = 1 sec) and HSQC experiments. For LC-MS analysis, a HPLC-system (detection simultaneously at 214 and 254 nm) equipped with an analytical C_{18} column (4.6 mmD x 250 mmL, 5μ particle size) in combination with buffers A: H_2O, B: CH_3CN and C: 1% aq TFA and coupled to a mass instrument with an electronspray interface (ESI) was used. For RP-HPLC purifications, an automated HPLC system equipped with a semi-preparative C_{18} column (5 μm C_{18}, 10Å, 150 x 21.2 mm) was used.

The applied buffers were A: H_2O + ammonium acetate (20 mM) and B: CH_3CN. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60000 at *m/z* 400 (mass range *m/z* = 150-2000) and dioctylpthalate (*m/z* = 391.28428) as a lock mass. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). Optical rotations were measured on a Propol automatic polarimeter (Sodium D-line, λ = 589 nm). ATR-IR spectra were recorded on a Shimadzu FTIR-8300 fitted with a single bounce Durasample IR diamond crystal ATR-element and are reported in cm^{-1}.

2,3,5-Tri-*O*-benzyl-L-ribitol (7). To a cooled solution (0 °C) of 2,3,5-tri-*O*-benzyl-L-ribofuranose (**6**) (9.97 g, 23.6 mmol)[10] in EtOH (197 mL) was added sodium borohydride (2.07 g, 54.5 mmol). After 3 h at room temperature, TLC analysis showed complete conversion of the starting material into a lower running product and the pH of the reaction mixture was adjusted to 5 by addition of acetic acid. The resulting mixture was concentrated, dissolved in EtOAc (300 mL) and washed consecutively with 1M aq HCl (200 mL), sat aq $NaHCO_3$ (200 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$), concentrated and the residue was purified by silica gel column chromatography (20 to 60% EtOAc in PE) to give **7** (9.56 g, 22.7 mmol) as a colorless oil in 96% yield. R_f = 0.37 (50% EtOAc in PE). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.20 (m, 15H, H_{ar} Bn), 4.70 (d, $J$$H_a$-$H_b$ = 11.2, 1H, C*H*H Bn), 4.70 (d, $J$$H_a$-$H_b$ = 11.7, 1H, C*H*H Bn), 4.57 (d, $J$$H_b$-$H_a$ = 11.7, 1H, CH*H* Bn), 4.56 (d, $J$$H_b$-Ha = 11.2, 1H, CH*H* Bn), 4.49 (d, $J$$H_a$-$H_b$ = 11.9, 1H, C*H*H Bn), 4.46 (d, $J$$H_b$-$H_a$ = 11.9, 1H, CH*H* Bn), 3.99 (m, 1H, H-4), 3.86 – 3.74 (m, 4H, H-1_a, H-1b, H-2, H-3), 3.60-3.54 (m, 2H, H-5_a, H-5_b), 3.05 (br s, OH), 2.76 (br s, OH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 137.9 (2 × C_q Bn), 137.7 (C_q Bn), 128.3, 128.4, 128.3, 128.1, 127.9, 127.8, 127.7, 127.6 (9 × CH_{ar} Bn), 79.3, 79.2 (C-2, C-3), 73.8, 73.3, 71.9 (3 × CH_2 Bn), 71.0 (C-5), 70.4 (C-4), 60.9 (C-1). ATR-IR (thin film) 3418, 2866, 1497, 1454, 1362, 1312, 1207, 1099, 1065, 1026, 910, 822, 733, 694 cm^{-1}. [α]D^{20}: -16.9° (c = 2.3, $CHCl_3$). ESI-MS *m/z*: obsd 423.1 $[M + H]^+$, 845.4 $[2M + H]^+$. HRMS *m/z*: calcd for $C_{26}H_{31}O_5$ $+H^+$: 423.21660; obsd 423.21672.

2,3,5-Tri-*O*-benzyl-1-*O*-trityl-L-ribitol (8). Compound **7** (9.56 g, 22.7 mmol) was coevaporated three times with DCE and dissolved in DCM (130 mL). To this solution was added Et_3N (6.52 mL, 46.8 mmol), triphenylmethyl chloride (8.7 g, 31.2 mmol) and DMAP (318 mg, 2.6 mmol). The reaction mixture was stirred for 16 h after which it was quenched by addition of MeOH (1 mL). The volatiles were removed, the residue was dissolved in EtOAc (300 mL) and washed successively with 0.1M aq HCl (200 mL), sat aq $NaHCO_3$ (200 mL) and brine (150 mL). The organic phase was dried ($MgSO_4$), concentrated and the residue purified by silica gel column chromatography (5 to 40% EtOAc in PE) to yield **8** as a colorless oil (14.02 g, 21.1 mmol) in 93%. R_f = 0.88 (50% EtOAc in PE). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.48 – 7.02 (m, 30H, H_{ar} Bn, Tr), 4.76 (d, $J$$H_a$-$H_b$ = 11.6, 1H, C*H*H Bn), 4.54 (d, $J$$H_b$-$H_a$ = 11.6, 1H, CH*H* Bn), 4.51 (s, 2H, CH_2 Bn), 4.46 (s, 2H, CH_2 Bn), 4.00 (m, 1H, H-4), 3.85 – 3.84 (m, 2H, H-2, H-3), 3.57 – 3.53 (m, 3H, H-5_a, H-5_b, H-1_a), 3.05 (m, 1H, H-1_b), 2.81 (br s, OH-4). ^{13}C NMR (100 MHz, $CDCl_3$) δ = 143.9 (3 × C_q Tr), 138.2, 138.1, 137.9 (3 × C_q Bn), 128.7, 128.3, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.5, 127.4, 126.9 (12 × CH_{ar} Bn, Tr), 86.8 (C_q Tr), 79.4, 78.9 (C-2, C-3), 73.6, 73.2, 72.5 (3 × CH_2 Bn), 71.2 (C-5), 71.0 (C-4), 63.1 (C-1). ATR-IR (thin film) 3043, 2893, 1493, 1450, 1327, 1211, 1072, 1026, 1003, 903, 733, 698, 633, 613 cm^{-1}. [α]D^{20}: -20.0° (c = 6.5, CHCl3). ESI-MS *m/z*: obsd 687.6 $[M + Na]^+$.

2,3,5-Tri-*O*-benzyl-4-methanesulfonyl-1-*O*-trityl-L-ribitol (9).Compound **8** (14.94 g, 22.5 mmol) was coevaporated three times with toluene, dissolved in pyridine (50 mL) and cooled (0 °C). The solution was charged with methanesulfonylchloride (4.6 mL, 57.5 mmol) and stirred for 16 h at 5 °C. The reaction mixture was

quenched by addition of MeOH (5 mL) and concentrated. The residue was dissolved in EtOAc (200 mL) and washed successively with 0.1M aq HCl (150 mL), sat aq $NaHCO_3$ (150 mL) and brine (100 mL). The organic phase was dried ($MgSO_4$), concentrated and the residue was purified by silica gel column chromatography (50 to 100% toluene in PE) to afford **9** as a colorless oil (16.42 g, 22.1 mmol) in 98% yield. *R*f = 0.49 (10% EtOAc in toluene). ^{1}H NMR (300 MHz $CDCl_3$) δ 7.48 – 7.04 (m, 30H, H_{ar} Bn, Tr), 4.23 (dt, *J*H4-H3 = 7.7, *J*H4-H5 = 2.7, 1H, H-4), 4.73 (d, JH_a-H_b = 11.6, 1H, C*H*H Bn), 4.66 (d, JH_a-H_b = 10.9, 1H, C*H*H Bn), 4.46 – 4.38 (m, 4H, CH_2 Bn, 2 × CH*H* Bn), 4.11 (dd, *J*H3-H4 = 7.7, *J*H3-H2 = 2.2, 1H, H-3), 3.80 – 3.61 (m, 3H, H-2, H-5_a, H-5_b), (dd, $JH1_a$-$H1_b$ = 10.3, *J*H1a-H2 = 2.4, 1H, H-1_a), 3.21 (dd, $JH1_b$-$H1_a$ = 10.3, $JH1_b$-H2 = 4.5 1H, H-1_b), 2.90 (s, 3H, CH_3 Ms). ^{13}C NMR (75 MHz, $CDCl_3$) δ 143.8 (3 × C_q Tr), 137.8, 137.6, 137.4 (3 × C_q Bn), 128.7, 128.3, 128.2, 128.0, 127.9, 127.8, 127.7, 127.6, 126.9 (12 × CH_{ar} Bn, Tr), 86.7 (C_q Tr), 82.9 (C-4), 78.6, 77.6 (C-2, C-3), 73.8, 73.1, 72.1 (3 × CH_2 Bn), 68.8 (C-5), 62.5 (C-1), 38.3 (CH_3 Ms). ATR-IR (thin film) 3030, 2875, 1490, 1448, 1356, 1333, 1217, 1175, 1076, 1028, 968, 912, 810, 745, 696, 667, 633 cm^{-1}. [α]D^{20}: -26.2° (c = 1.8, $CHCl_3$). ESI-MS *m/z*: obsd 765.2 $[M + Na]^+$.

4-Azido-2,3,5-tri-*O*-benzyl-4-deoxy-1-*O*-trityl-D-lyxitol (10). Mesylate **9** (15.37 g, 20.9 mmol) was coevaporated three times with toluene and dissolved in DMF (112 mL). To the solution of mesylate 4 were added sodium azide (8.71 g, 134 mmol), 15-crown-5 (0.89 mL, 4.46 mmol) and tetrabutylammonium hydrogensulfate (1.514 g, 4.46 mmol). The resulting suspension was stirred at 90 °C for 48 h. The reaction mixture was concentrated. The residue was dissolved in EtOAc (200 mL) and washed successively with water (200 mL), sat aq $NaHCO_3$ (200 mL) and brine (100 mL). The organic layer was dried ($MgSO_4$), concentrated and the resulting residue was purified by silica gel column chromatography (5 to 20% EtOAc in PE) to produce **10** as off-white crystals (13.16 g, 19.1 mmol) in 94% yield. R_f = 0.49 (20% EtOAc in PE). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.50 – 6.94 (m, 30H, H_{ar} Bn, Tr), 4.78 (d, JH_a-H_b = 11.3, 1H, C*H*H Bn), 4.54 – 4.48 (m, 3H, CH_2 Bn, C*H*H Bn), 4.39 (m, 2H, CH_2 Bn), 4.02 – 3.89 (m, 2H, H-2, H-3), 4.75 (m, 1H, H-4), 3.69 – 3.57 (m, 3H, H-5_a, H-5_b, H-1_a), 3.21 (m, 1H, H-1b). ^{13}C NMR (50 MHz, $CDCl_3$) δ 143.8 (3 × C_q Tr), 137.9 (C_q Bn), 137.6 (2 × C_q Bn), 128.7, 128.3, 128.1, 127.7, 126.9 (12 × CH_{ar} Bn, Tr), 86.6 (C_q Tr), 78.3, 77.0 (C-2, C-3), 74.2, 73.2, 72.6 (3 × CH_2 Bn), 69.5 (C-5), 61.9 (C-1), 60.9 (C-4). ATR-IR (thin film) 3036, 2901, 2095, 1593, 1493, 1450, 1331, 1265, 1215, 1092, 999, 907, 733, 694, 633 cm^{-1}. [α]D^{20}: -25.6° (c = 0.9, $CHCl_3$). ESI-MS *m/z*: obsd 712.5 $[M + Na]^+$.

4-Azido-2,3,5-tri-*O*-benzyl-4-deoxy-D-lyxitol (11). Azide **10** (13.15 g, 19.1 mmol) was dissolved in a mixture of chloroform (96 mL) and MeOH (96 mL), a catalytic amount of *p*-toluenesulfonic acid (183 mg, 0.96 mmol) was added. After stirring for 16 h, the reaction was quenched by addition of Et_3N (0.5 mL) and concentrated. The residue was dissolved in EtOAc, cooled to 0 °C and the triphenylmethoxymethane that precipitated was removed by filtration. Evaporation of the volatiles and purification of the residue by silica gel column chromatography (20 to 33% EtOAc in PE) afforded **11** as a colorless oil (7.56 g, 16.9 mmol) in 88% yield. R_f = 0.40 (20% EtOAc in toluene). ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.33 – 7.24 (m, 15H, H_{ar} Bn), 4.67 (d, JH_a-H_b = 11.3, 1H, CHH Bn), 4.57 (d, JH_a-H_b = 11.3, 1H, C*H*H Bn), 4.52 – 4.44 (m, 3H, CH*H* Bn, 2 × C*H*H Bn), 4.42 (d, JH_b-H_a = 11.9, 1H, CH*H* Bn), 3.85 (dd, *J*H1a-H2 = 3.0, *J*H1a-H1b = 12.2, 1H, H-1a), 3.80 – 3.77 (m, 2H, H-3, H-4), 3.69 (dd, *J*H1b-H2 = 2.6, $JH1_b$-$H1_a$ = 12.2, 1H, H-1b), 3.66 – 3.62 (m, 2H, H-5a, H-2), 3.53 (dd, $JH5_b$-H4 = 5.6, $JH5_b$-$H5_a$ = 9.7, 1H, H-5b), 2.19 (br s, 1H, OH-1). ^{13}C NMR (50 MHz, $CDCl_3$) δ = 137.5 (2 × C_q Bn), 137.3 (C_q Bn), 128.4, 128.3, 128.2, 127.8, 127.7, 127.6, 127.5 (9 × CH_{ar} Bn), 78.5 (C-2), 76.5 (C-3), 74.4, 73.2, 72.0 (3 × CH_2 Bn), 69.4 (C-5), 60.9 (C-4), 59.3 (C-1). ATR-IR (thin film) 3364, 2866, 2098, 1454, 1331, 1273, 1095, 1053, 1026, 737, 698 cm^{-1}. [α]D^{20}: -20.6° (c = 0.7, $CHCl_3$). ESI-MS *m/z*: obsd 448.1 $[M + H]^+$, 470.4 $[M + Na]^+$. HRMS *m/z*: calcd for $C_{26}H_{30}N_3O_4$ $+H^+$: 448.22308; obsd 448.22311.

4-Azido-2,3,5,-tri-*O*-benzyl-4-deoxy-D-lyxose (1). Compound **11** (3.11 g, 6.96 mmol) was coevaporated three times with DCE and subsequently dissolved in DCM (12 mL). The solution was cooled (0 °C) and Dess-Martin periodinane (3.55 g, 8.35 mmol) was added. The reaction mixture was stirred for 3 h at 0 °C, during which a white suspension formed. A mixture of sat aq $NaHCO_3$ and 1M $Na_2S_2O_3$ (1/1 v/v, 100 mL) was added to the suspension, and vigorously stirred for 5 min. EtOAc (100 mL) was added to the mixture and the separated organic phase was washed three times with brine (100 mL). The organic layer was dried ($MgSO_4$), concentrated and the residue was purified by silica gel column chromatography (10 to 33% EtOAc in PE) to provide 4azidopentanal **1** (3.10 g, 6.96 mmol) as a colorless oil in quantitative yield. R_f = 0.68 (20% EtOAc in toluene). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.59 (d, *J*H1-H2 = 1.0, 1H, H-1), 7.29 – 7.24 (m, 15H, H_{ar} Bn), 4.64 (d, *J*H_a-H_b = 11.6, 1H, C*H*H Bn), 4.55 (d, *J*H_a-H_b = 11.3, 1H, C*H*H Bn), 4.51 – 4.48 (m, 2H, 2 × CH*H* Bn), 4.42 (d, *J*H_a-H_b = 11.9, 1H, C*H*H Bn), 4.37 (d, *J*H_b-H_a = 11.9, 1H, CH*H* Bn), 4.01 (dd, *J*H2-H1 = 1, *J*H2-H3 = 4.2, 1H, H-2), 3.96 (dd, *J*H3-H2 = *J*H3-H4 = 4.8, 1H, H-3), 3.80 – 3.77 (m, 1H, H-4), 3.64 – 3.55 (m, 2H, H-5_a, H-5_b). ^{13}C NMR (50 MHz, $CDCl_3$) δ 200.4 (C-1), 137.2, 136.9, 136.7 (3 × C_q Bn), 128.3, 128.2, 127.9, 127.7, 127.6 (9 × CH_{ar} Bn), 82.8 (C-2), 78.7 (C3), 73.8, 73.0, 72.8 (3 × CH_2 Bn), 68.8 (C-5), 61.4 (C-4). ATR-IR (thin film) 2868, 2098, 1732, 1454, 1325, 1267, 1209, 1096, 1051, 1028, 910, 737, 698 cm^{-1}. [α]D^{20}: -26.0° (c = 8.5, $CHCl_3$). ESI-MS *m/z*: obsd 446.2 $[M + H]^+$, 468.1 $[M + Na]^+$. HRMS *m/z*: calcd for $C_{26}H_{28}N_3O_4 + H^+$: 446.20743; obsd 446.20747.

***tert*-Butyl 3-phenylprop-2-ynylcarbamate ([0,1]-12)**. In separate flasks, a solution of *N*-Boc-propargyl amine (0.93 g, 6.0 mmol), iodobenzene (1.02 g, 5.0 mmol) and pyrrolidine (1.96 mL, 24 mmol) in DMF (20 mL), a solution of CuI (96 mg, 0.5 mmol) in DMF (1 mL) and a solution of $Pd(PPh_3)_4$ (289 mg, 0.25 mmol) in DMF (1 mL) were flushed with argon for 1 h in an ultrasonic bath. To the alkyne solution were added subsequently the CuI and the $Pd(PPh_3)_4$ solutions and the mixture was stirred for 18 h under inert atmosphere. The volatiles were removed and the crude product dissolved in EtOAc (100 mL) and washed with saturated aqueous NH_4Cl solution (3 × 50 mL) and 10% aqueous $NaHCO_3$ (50 mL). The organic layer was dried with Na_2SO_4 and concentrated. The crude material was purified by silica gel column chromatography (0 to 10% EtOAc/toluene) to yield titled compound (1.1 g, 4.8 mmol) in 96% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 – 7.39 (m, 2H, 2 × H Ar), 7.31 – 7.27 (m, 3H, 3 × H Ar), 4.82 (s, 1H, NH), 4.15 (d, *J* = 4.0, 2H, CH_2) 1.47 (s, 9H, 3 × CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.3 (C), 131.7 (2 × CH Ar), 128.3 (CH Ar), 128.3 (2 × CH Ar), 122.7 (C Ar), 85.4 (C), 83.1 (C), 80.0 (C), 31.2 (CH_2), 28.4 (3 × CH_3).

General procedure for coupling of N-Boc-propargyl amine with 1,2-diiodobenzene (affording [1,2]-12), 1,3-diiodobenzene (affording [1,3]-12), and 1,4-diiodobenzene (affording [1,4]-12).
In separate flasks, a solution of *N*-Boc-propargyl amine (930 mg, 6.0 mmol), the desired diiodobenzene (0.83 g, 2.5 mmol) and pyrrolidine (4.9 mL, 60 mmol,) in DMF (50 mL), a solution of CuI (96 mg, 0.5mmol) in DMF (1 mL) and a solution of $Pd(PPh_3)_4$ (289 mg, 0.5 mmol) in DMF (1 mL) were flushed with argon for 1 h in an ultrasonic bath. To the alkyne solution were added subsequently the CuI and the $Pd(PPh_3)_4$ solutions and the mixture were stirred for 18 h under argon atmosphere. The volatiles were removed and the crude product dissolved in EtOAc (100 mL) and washed with saturated aqueous NH_4Cl solution (3 × 50 mL) and 10% aq $NaHCO_3$ (50 mL). The organic layer was dried with Na_2SO_4 and concentrated. The crude material was purified by silica gel column chromatography (0 to 10% EtOAc in toluene).

***tert*-Butyl 3,3'-(1,2-phenylene)bis(prop-2-yne-3,1-diyl)dicarbamate ([1,2]-12)**. Yield: 0.71 g (1.8 mmol, 73%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 – 7.36 (m, 2H, 2 × H Ar), 7.26 – 7.20 (m, 2H, 2 × H Ar), 5.29 (br s, 2H, NH), 4.19 (d, *J* = 3.8, 4H, 2 × CH_2) 1.47 (s, 18H, 6 × CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.6 (2 × C), 131.7 (2 × CH Ar), 128.3 (2 × CH Ar), 125.6 (2 × C Ar), 89.8 (2 × C), 81.5 (2 × C), 80.0 (2 × C), 31.3 (2 × CH_2), 28.6 (6 × CH_3).

***tert*-Butyl 3,3'-(1,3-phenylene)bis(prop-2-yne-3,1-diyl)dicarbamate ([1,3]-12)**. Yield: 0.95 g (2.5 mmol, 99%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 (s, 1H, H Ar), 7.36 – 7.29 (m, 2H, 2 × H Ar), 7.25 – 7.19 (m, 1H, H Ar), 5.05 (br s, 2H, NH), 4.14 (d, *J* = 4.6, 4H, 2 × CH_2) 1.47 (s, 18H, 6 × CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.3 (2 × C), 134.4 (1 × CH Ar), 131.1 (2 × CH Ar), 128.0 (1 × CH Ar), 122.7 (2 × C Ar), 86.0 (2 × C), 81.8 (2 × C), 79.7 (2 × C), 30.4 (2 × CH_2), 28.0 (6 × CH_3).

***tert*-Butyl 3,3'-(1,3-phenylene)bis(prop-2-yne-3,1-diyl)dicarbamate ([1,4]-12)**. Yield: 0.87 g (2.3 mmol, 91%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 (s, 4H, 4 × H Ar), 4.86 (br s, 2H, NH), 4.15 (d, *J* = 3.3, 4H, 2 × CH_2) 1.47 (s, 18H, 6 × CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.3 (2 × C), 131.6 (4 × CH Ar), 122.7 (2 × C Ar), 87.3 (2 × C), 82.6 (2 × C), 80.0 (2 × C), 31.2 (2 × CH_2), 28.3 (6 × CH_3).

***N*-(3-Phenylprop-2-ynyl)formamide ([0,1]-14)**. Boc protected compound **[0,1]-12** (460 mg, 2.0 mmol) was subjected to 50 mL of a DCM/TFA (4/1; v/v) solution for 18h. When the reaction was complete, 25 mL of toluene was added and the solvents were evaporated. The crude product was coevaporated three times with dry toluene. The crude free amine was dissolved in ethanol (20 mL), ethyl formate (20 mL) and triethyl amine (0.5 mL) and heated to reflux for 18 h. The mixture was concentrated and the residue was purified by silica gel column chromatography (0 to 10% methanol/EtOAc) to afford 202 mg (1.3 mmol, 64%) of the titled compound. ^{1}H NMR (400 MHz, MeOD) δ 8.11 (s, 1H, CHO), 7.42 – 7.36 (m, 2H, 2 × H Ar), 7.32 – 7.26 (m, 3H, 3 × H Ar), 4.22 (s, 2H, CH_2). ^{13}C NMR (100 MHz, MeOD) δ 163.4 (CHO), 132.7 (2 × CH Ar), 129.6 (CH Ar), 129.5 (2 × CH Ar), 124.1 (C Ar), 85.7 (C), 83.7 (C), 28.9 (CH_2).

General procedure for the preparation of dimeric formamides [1,2]-14, [1,3]-14 and [1,4]-14.
Boc protected compounds **[1,2]-12, [1,3]-12** or **[1,4]-12** (384 mg, 1.0 mmol) was subjected to 50 mL of a DCM/TFA (4/1; v/v) solution for 18 h. When the reaction was complete, 25 mL of toluene was added and the solvents were evaporated. The crude product was coevaporated three times with dry toluene. The crude free amine was dissolved in ethanol (20 mL), ethyl formate (20 mL) and triethyl amine (0.5 mL) and heated to reflux for 18h. The mixture was concentrated and the residue was purified by silica gel column chromatography (0 to 10% methanol in EtOAc) to afford the titled compounds.

***N,N'*-(3,3'-(1,2-Phenylene)bis(prop-2-yne-3,1-diyl))diformamide ([1,2]-14)**. Yield: 182 mg (0.76 mmol, 76%). ^{1}H NMR (400 MHz, MeOD) δ 8.10 (s, 2H, CHO), 7.46 – 7.36 (m, 2H, 2 × H Ar), 7.35 – 7.21 (m, 2H, 2 × H Ar), 4.29 (s, 4H, 2 × CH_2). ^{13}C NMR (100 MHz, MeOD) δ 163.6 (2 × CHO), 133.1 (2 × CH Ar), 129.4 (2 × CH Ar), 126.8 (2 × C Ar), 90.0 (2 × C), 82.1 (2 × C), 29.0 (2 × CH_2).

***N,N'*-(3,3'-(1,3-Phenylene)bis(prop-2-yne-3,1-diyl))diformamide ([1,3]-14)**. Yield: 192 mg (0.80 mmol, 80%). ^{1}H NMR (400 MHz, MeOD) δ 8.10 (s, 2H, CHO), 7.42 (s, 1H, H Ar), 7.39 – 7.33 (m, 2H, 2 × H Ar), 7.31 – 7.25 (m, 1H, H Ar), 4.22 (s, 4H, 2 × CH_2). ^{13}C NMR (100 MHz, MeOD) δ 163.5 (2 × CHO), 135.6 (1 × CH Ar), 132.7 (2 × CH Ar), 129.9 (1 × CH Ar), 124.6 (2 × C Ar), 86.6 (2 × C), 82.6 (2 × C), 28.9 (2 × CH_2).

***N,N'*-(3,3'-(1,4-Phenylene)bis(prop-2-yne-3,1-diyl))diformamide ([1,4]-14)**. Yield: 166 mg (0.69 mmol, 69%). ^{1}H NMR (400 MHz, MeOD) δ 8.10 (s, 2H, CHO), 7.37 (s, 4H, H Ar), 4.25 (s, 4H, 2 × CH_2). ^{13}C NMR (100 MHz, MeOD) δ 163.5 (2 × CHO), 132.8 (4 × CH Ar), 124.3 (2 × C Ar), 87.7 (2 × C), 83.0 (2 × C), 28.9 (2 × CH_2).

(3-Isocyanoprop-1-ynyl)benzene ([0,1]-5). Phorphorylchloride (0.84 mL, 0.9 mmol) was added dropwise to a dry and cooled (-30 °C) solution of formamide **[0,1]-14** (95 mg, 0.6 mmol) and Et3N (0.42 mL, 9 mmol) in

DCM (5 mL). The reaction mixture was stirred for 1 h at -30 °C, after which TLC analysis indicated complete consumption of the formamide. The reaction mixture was quenched by addition of sat aq $NaHCO_3$ (1 mL). The reaction mixture was diluted with DCM (25 mL) and washed successively with sat aq $NaHCO_3$ (2 × 10 mL) and brine (10 mL). The organic phase was dried (Na_2SO_4) and concentrated. The resulting residue was purified by flushing over a plug of silicagel ($CHCl_3$) to afford isocyanide **[0,1]-5** (55 mg, 0.42 mmol) in 81% yield as a colorless oil that turns brown on standing. The isocyanide was used immediately in the SAWU-MCR.

General procedure for the preparation of dimeric isocyanides [1,2]-5, [1,3]-5 and [1,4]-5.
Phorphorylchloride (0.84 mL, 0.9 mmol) was added dropwise to a dry and cooled (−30 °C) solution of formamide **[0,1]-14** (72 mg, 0.3 mmol) and Et_3N (0.42 mL, 9 mmol) in DCM (5 mL). The reaction mixture was stirred for 1 h at −30 °C, after which TLC analysis indicated complete consumption of the formamide. The reaction mixture was quenched by addition of sat aq $NaHCO_3$ (1 mL).The reaction mixture was diluted with DCM (25 mL) and washed successively with sat aq $NaHCO_3$ (2 × 10 mL) and brine (10 mL). The organic phase was dried (Na_2SO_4) and concentrated. The resulting residue was purified by flushing over a plug of silicagel ($CHCl_3$) to afford the dimeric isocyanide as colorless oil.

1,2-Bis(3-isocyanoprop-1-ynyl)benzene ([1,2]-5). Yield: 24 mg (0.11 mmol, 39%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (dd, *J* = 3.4, 5.7, 2H, H Ar), 7.34 (dd, *J* = 3.4, 5.8, 2H, 2 × H Ar), 4.53 (s, 4H, 2 × CH_2). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.6 (2 × NC), 132.0 (2 × CH Ar), 129.0 (2 × CH Ar), 122.0 (2 × C Ar), 84.1 (2 × C), 79.6 (2 × C), 32.3 (2 × CH_2).

1,3-bis(3-isocyanoprop-1-ynyl)benzene ([1,3]-5). Yield: 45 mg (0.22 mmol, 73%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 (s, 1H, H Ar), 7.46 – 7.44 (m, 2H, 2 × H Ar), 7.34 – 7.30 (m, 1H, H Ar), 4.46 (s, 4H, 2 × CH_2). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.6 (2 × NC), 135.0 (1 × CH Ar), 132.4 (2 × CH Ar), 128.7 (1 × CH Ar), 122.0 (2 × C Ar), 84.1 (2 × C), 79.6 (2 × C), 32.3 (2 × CH_2).

1,4-bis(3-isocyanoprop-1-ynyl)benzene ([1,4]-5). Yield: 62 mg (0.29 mmol, 97%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 (s, 4H, 4 × H Ar), 4.47 (s, 4H, 2 × CH_2). ^{13}C NMR (101 MHz, $CDCl_3$) δ 158.6 (2 × NC), 131.9 (4 × CH Ar), 122.2 (2 × C Ar), 84.5 (2 × C), 80.7 (2 × C), 32.4 (2 × CH_2).

General procedure for the SAWU-3CR towards substituted pyrrolidines [0,1]-15, [1,2]-15, [1,3]-15 and [1,4]-15.
4-azidopentanal (**1**) was coevaporated with toluene, dissolved in MeOH (0.05 M) and cooled (0 °C). After dropwise addition of a solution of trimethylphosphine (1M in toluene, 2 eq), stirring was continued for 3 hours at 0 °C when TLC analysis indicated complete consumption of the 4-azidopentanal and the appearance of the intermediate phosphazene (R_f = 0 in 50% EtOAc in toluene). The reaction mixture was concentrated and coevaporated with toluene, after which TLC analysis showed complete disappearance of the baseline phosphazene intermediate and emergence of a the cyclic imine. Formation of the pyrroline **2** was confirmed by NMR analysis of the crude product. (3R,4S,5R)-3,4-di-*O*-benzyl-5-benzyloxymethyl-1-pyrroline (**2**). R_f = 0.34 (20% EtOAc in toluene). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.67 (s, 1H, H-2), 7.32 – 7.26 (m, 15H, CH_{ar} Bn), 4.72 – 4.50 (m, 7H, 3 × CH_2 Bn, H-3), 4.35 (d, *J* = 4.7, 1H, H-4), 4.25 – 4.19 (m, 1H, H-5), 3.99 – 3.85 (m, 2H, H-6_a, H-6_b). ^{13}C NMR (50 MHz, $CDCl_3$) δ 166.9 (C-2), 138.0, 137.9, 137.2 (3 × C Ar), 128.2, 127.9, 127.6, 127.4, 127.3 (9 × CH Ar), 85.6, 77.7, 73.2 (C-3, C-4, C-5), 73.4, 73.1, 72.7 (3 × CH_2 Bn), 68.5 (C-6). The crude pyrroline (**2**) was dissolved in MeOH (0.1M), divided in portions and cooled (0 °C). The carboxylic acid (1 eq for the monomers and 2 eq for dimers) and isocyanide (1 eq for the monomers and 2 eq for dimers) were added. The reaction mixture was stirred for 2 hours

during which it was allowed to warm to room temperature. The reaction mixtures were concentrated and the product was isolated by size exclusion chromatography (DCM/MeOH) and silica gel column chromatography (1 to 3% MeOH/DCM) to afford the SAWU-3CR products as yellow solids.

Monomeric ligand [0,1]-15. Yield: 45 mg (46 µmol, 46%). LC-MS analysis: *t*R 12.8 min (gradient 10 to 90% B). ESI-MS *m/z*: 988.3 $[M + H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 – 7.10 (m, 21H), 6.84 (d, *J* = 7.4, 1H), 6.71 (d, *J* = 7.9, 1H), 6.65 (s, 1H), 6.03 (br s, 2H), 5.21 (s, 1H), 4.79 – 4.75 (m, 2H), 4.66 (d, *J* = 11.5, 1H), 4.61– 4.51 (m, 3H), 4.50 – 4.40 (m, 3H), 4.40 – 4.26 (m, 2H), 4.23 (t, *J* = 10.2, 1H), 4.15 – 3.90 (m, 2H), 3.89 – 3.76 (m, 2H), 2.61 (s, 3H), 1.43 (s, 9H). HRMS m/z calcd for $C_{56}H_{57}N_7O_6S_2 + H^+$: 988.38845, obsd 988.38738.

Dimeric ligand [1,2]-15. Yield: 31 mg (16 µmol, 32%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 – 6.90 (m, 36H), 6.77 (d, *J* = 7.3, 2H), 6.64 (d, *J* = 8.0, 2H), 6.60 (s, 2H), 6.02 (br s, 4H), 5.21 (s, 2H), 4.80 – 4.63 (m, 6H), 4.61 – 4.31 (m, 16H), 4.29 – 4.10 (m, 4H), 4.10 – 3.74 (m, 6H), 2.57 (s, 6H), 1.43 (s, 18H). HRMS m/z calcd for $C_{106}H_{108}N_{14}O_{12}S_4 + H^+$: 1897.72267, obsd 1897.71509, calcd for $C_{106}H_{108}N_{14}O_{12}S_4 + 2H^+$: 949.36498, obsd 949.36601.

Dimeric ligand [1,3]-15. Yield: 36 mg (19 µmol, 38%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 – 7.00 (m, 36H), 6.82 (d, *J* = 6.9, 2H), 6.74 – 6.59 (s, 4H), 6.01 (br s, 4H), 5.22 (s, 2H), 4.79 – 4.60 (m, 6H), 4.60 – 4.26 (m, 16H), 4.25 – 4.08 (m, 4H), 4.08 – 3.74 (m, 6H), 2.60 (s, 6H), 1.43 (s, 18H). HRMS m/z calcd for $C_{106}H_{108}N_{14}O_{12}S_4 + H^+$: 1897.72267, obsd 1897.73181, calcd for $C_{106}H_{108}N_{14}O_{12}S_4 + 2H^+$: 949.36498, obsd 949.36569.

Dimeric ligand [1,4]-15. . Yield: 46 mg (24 µmol, 48%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39 – 6.94 (m, 36H), 6.84 (d, *J* = 7.2, 2H), 6.79 – 6.56 (s, 4H), 6.03 (br s, 4H), 5.21 (s, 2H), 4.90 – 4.62 (m, 6H), 4.61 – 4.26 (m, 16H), 4.26 – 4.07 (m, 4H), 4.05 – 3.77 (m, 6H), 2.61 (s, 6H), 1.43 (s, 18H). HRMS m/z calcd for $C_{106}H_{108}N_{14}O_{12}S_4 + 2H^+$: 949.36498, obsd 949.36590.

General procedure for benzyl cleavage towards substituted hydroxyprolines [0,1]-hyp-LHA, [1,2]-hyp-LHA$_2$, [1,3]-hyp-LHA$_2$ and [1,4]-hyp-LHA$_2$.

Boron trichloride (10 eq per benzyl, 1M in DCM) was added to a cooled (0 °C) solution of the benzylated SAWU product **[0,1]-15** (70 µmol)**, [1,2]-15, [1,3]-15** or **[1,4]-15** (10 µmol) in DCM (0.05M). The reaction mixture was stirred for 20 hours at 0–5 °C after which MeOH (0.5 mL) was carefully added. The reaction mixture was concentrated and coevaporated with toluene (3×). The obtained residue was purified by preparative HPLC column chromatography (0 to 30% B) to yield the deprotected compounds as yellow solids.

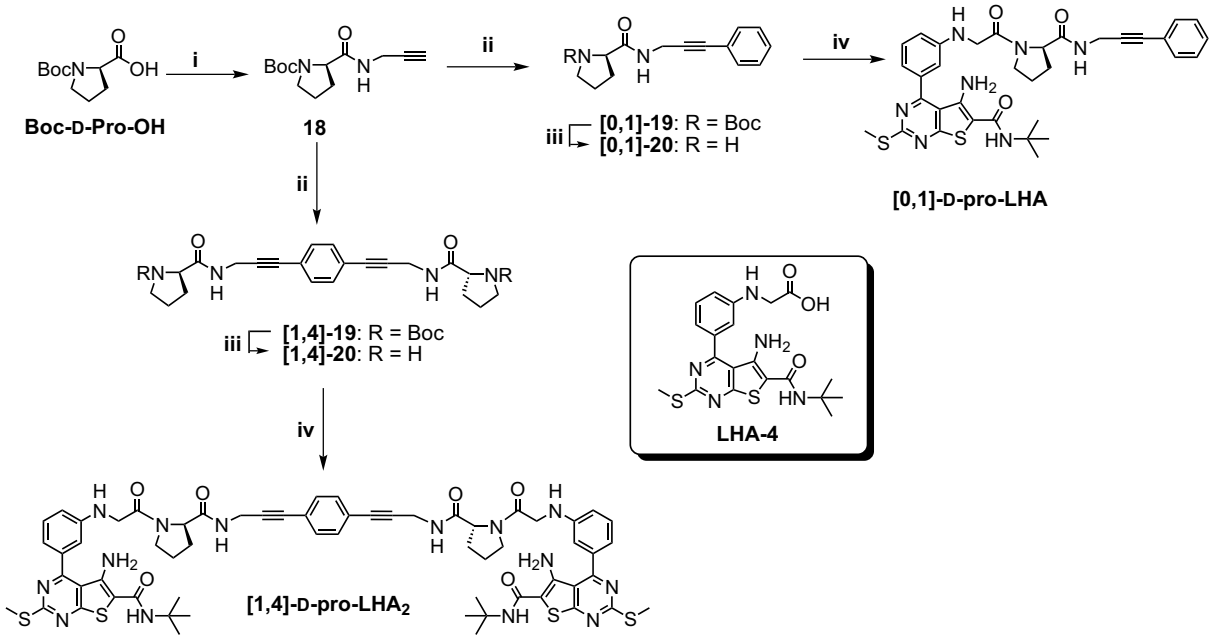
Monomeric ligand [0,1]-hyp-LHA. Yield after RP-HPLC purification: 4.5 mg (5.0 µmol, 7%). LC-MS analysis: t_R 9.0 min (gradient 10 to 90% B). ESI-MS *m/z*: 718.3 $[M + H]^+$. ^{1}H NMR (400 MHz, MeOD) δ 7.43 – 7.37 (m, 1H), 7.36 – 7.26 (m, 3H), 7.26 – 7.18 (m, 2H), 6.94 – 6.88 (m, 1H), 6.88 – 6.82 (m, 1H), 6.81 – 6.72 (m, 1H), 4.64 (t, *J* = 6.7, 1H), 4.44 – 4.39 (m, 1H), 4.36 (t, *J* = 5.3, 1H), 4.33 – 4.15 (m, 3H), 4.06 – 3.97 (m, 2H), 3.96 – 3.87 (m, 2H), 2.62 (s, 3H), 1.44 (s, 9H). HRMS m/z calcd for $C_{35}H_{39}N_7O_6S_2 + H^+$: 718.24760, obsd 718.24771.

Dimeric ligand [1,2]-hyp-LHA$_2$. Yield after RP-HPLC purification: 0.8 mg (0.5 µmol, 5%). LC-MS analysis: t_R 10.3 min (gradient 10 to 90% B). ESI-MS *m/z*: 1357.3 $[M + H]^+$. ^{1}H NMR (400 MHz, MeOD) δ 7.34 – 7.23 (m, 2H), 7.21 – 7.13 (m, 2H), 7.11 – 7.04 (m, 2H), 6.98 – 6.88 (m, 2H), 6.87 – 6.81 (m, 2H), 6.80 – 6.72 (m, 2H), 4.65 – 4.58 (m, 2H), 4.43 – 4.14 (m, 10H), 4.10 – 3.86 (m, 8H), 2.64 (s, 6H), 1.46 (s, 18H). HRMS m/z calcd for $C_{74}H_{72}N_{14}O_{12}S_4 + H^+$: 1357.44097, obsd 1357.44256.

Dimeric ligand [1,3]-hyp-LHA$_2$. Yield after RP-HPLC purification: 1.7 mg (1.1 μmol, 11%). LC-MS analysis: t_R 9.7 min (gradient 10 to 90% B). ESI-MS *m/z*: 1357.4 $[M + H]^+$. ^{1}H NMR (400 MHz, MeOD) δ 7.37 – 7.21 (m, 6H), 6.98 – 6.88 (m, 2H), 6.87 – 6.81 (m, 2H), 6.80 – 6.72 (m, 2H), 4.67 – 4.58 (m, 2H), 4.42 – 4.13 (m, 10H), 4.06 – 3.84 (m, 8H), 2.65 (s, 6H), 1.45 (s, 18H). HRMS m/z calcd for $C_{74}H_{72}N_{14}O_{12}S_4 + H^+$: 1357.44097, obsd 1357.44267.

Dimeric ligand [1,4]-hyp-LHA$_2$. Yield after RP-HPLC purification: 1.1 mg (0.7 μmol, 7%). LC-MS analysis: t_R 9.4 min (gradient 10 to 90% B). ESI-MS *m/z*: 1357.5 $[M + H]^+$. ^{1}H NMR (400 MHz, MeOD) δ 7.37 – 7.29 (m, 4H), 7.28 – 7.18 (m, 2H), 6.94 – 6.89 (m, 2H), 6.89 – 6.82 (m, 2H), 6.82 – 6.73 (m, 2H), 4.68 – 4.61 (m, 2H), 4.45 – 4.14 (m, 10H), 4.09 – 3.87 (m, 8H), 2.63 (s, 6H), 1.45 (s, 18H). HRMS m/z calcd for $C_{74}H_{72}N_{14}O_{12}S_4 + H^+$: 1357.44097, obsd 1357.44256.

*The linker systems for compounds **[0,1]-D-pro-LHA**, **[1,2]-D-pro-LHA**, **[1,3]-D-pro-LHA** and **[1,4]-D-pro-LHA** were prepared following the synthetic route as described in Chapter 3 (Scheme 1) and depicted below.*



Reagents and conditions: *i.* Isobutyl chloroformate, NMM, propargylamine, DCM, -20 °C to rt 18 h, 76%; *ii.* CuI, $Pd(PPh_3)_4$, pyrrolidine, DMF, 18 h; *iii.* TFA/DCM; 1/1; v/v, 1% TIS, 18 h, HPLC purification; *iv.* pharmacophore **LHA-4**, BOP, DiPEA, DMF, 18 h, HPLC purification.

***N*-α-*tert*-Boc-D-proline propargylamide (18)**. Isobutyl chloroformate (0.71 mL, 5.5 mmol) was added to a cooled (-20 °C) solution of Boc-D-Pro-OH (1.08 g, 5.0 mmol) and *N*-methylmorpholine (0.77 mL, 7.0 mmol) in DCM (50 mL). After stirring for 1 h, propargylamine (0.48 mL, 7.0 mmol) was added. The reaction mixture was warmed to room temperature over a period of 2 h and subsequently stirred for 16 h. The mixture was successively washed with 1M HCl (25 mL) and 10% aqueous $NaHCO_3$ (25 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated. The residue was dissolved in EtOAc and triturated with petroleum ether to afford titled product as white crystals (1.0 g, 4.0 mmol) in 80% yield. ^{1}H NMR mixture of rotamers (400 MHz, $CDCl_3$) δ 7.29 (br s, 0.5H), 6.29 (br s, 0.5H), 4.35 – 3.91 (br s, 3H), 3.57 – 3.25 (br s, 2H), 2.38 (br s, 0.5H), 2.30 – 2.04 (s, 2H), 2.04 – 1.77 (br s, 2.5H), 1.47 (s, 9H). ^{13}C NMR of rotamers (100 MHz, $CDCl_3$) δ 172.4, 80.7, 79.3, (3 × C), 70.3 (CH), 60.3, 59.5 (1 × CH), 46.8 (CH_2), 30.6, 28.5 (1 × CH_2), 28.5 (CH_2), 28.2 (3 × CH_3), 24.4, 23.8 (1 × CH_2).

***N*-α-*tert*-Boc-(3-phenylprop-2-yn-1-amide)-D-proline ([0,1]-19).** In separate flasks, a solution of the propargyl functionalized D-proline (**18**, 126 mg, 0.5 mmol), iodobenzene (155 mmol, 0.4 mmol) and pyrrolidine (2.4 mmol, 196 µL) in DMF (2 mL), a solution of CuI (7.7 mg, 0.04 mmol) in DMF (1 mL) and a solution of $Pd(PPh_3)_4$ (23.1 mg, 0.02 mmol) in DMF (1 mL) were flushed with argon for 1 h in an ultrasonic bath. To the alkyne solution were added subsequently the CuI and the $Pd(PPh_3)_4$ solutions and the mixture were stirred for 18 h under inert atmosphere. The volatiles were removed and the crude product dissolved in MeOH/DCM (1/9; v/v, 50 mL) and washed with water (3 × 25 mL) and 10% aqueous $NaHCO_3$ (25 mL). The organic layer was dried with Na_2SO_4 and concentrated. The crude material was purified by silica gel column chromatography (0 to 10% EtOAc in toluene) to yield titled compound (119 mg, 0.36 mmol) in 91% yield. ^{1}H NMR of rotamers (400 MHz, $CDCl_3$) δ 7.42 – 7.36 (m, 2H), 7.32 – 7.26 (m, 3H), 6.40 (br s, 1H), 4.45 – 4.13 (m, 3H), 3.55 – 3.27 (m, 2H), 2.35 – 1.82 (m, 5H), 1.46 (s, 9H). ^{13}C NMR of rotamers (100 MHz, $CDCl_3$) δ 172.5, 155.6 (2 × C), 131.6, 128.3 (2 × CH), 122.5, 84.6, 83.3, 80.5 (4 × C), 61.0, 59.8 (1 × CH), 47.0 (CH_2), 30.8, 29.7 (1 × CH_2), 29.7 (CH_2), 28.2 (3 × CH_3), 24.4, 23.7 (1 × CH_2).

General procedure for coupling of N-α-tert-Boc-D-proline propargylamide (18) with 1,2-diiodobenzene (affording [1,2]-19), 1,3-diiodobenzene (affording [1,3]-19), and 1,4diiodobenzene (affording [1,4]-19).

In separate flasks, a solution of propargyl functionalized Boc-D-proline (**18**, 252 mg, 1.0 mmol), the desired diiodobenzene (0.40 mmol, 132 mg) and pyrrolidine (4.80 mmol, 392 µL) in DMF (3 mL), a solution of CuI (0.1 mmol, 15.3 mg) in DMF (1 mL) and a solution of $Pd(PPh_3)_4$ (0.04 mmol, 46.2 mg) in DMF (1 mL) were was flushed with argon for 1 h in an ultrasonic bath. To the alkyne solution were added subsequently the CuI and the $Pd(PPh_3)_4$ solutions and the mixture were stirred for 18 h under argon atmosphere. The volatiles were removed and the crude product dissolved in MeOH/DCM (1/9; v/v, 50 mL) and washed with water (3 × 25 mL) and 10% aqueous $NaHCO_3$ (25 mL). The organic layer was dried with Na_2SO_4 and concentrated. The crude material was purified by silica gel column chromatography (50 to 100% EtOAc in toluene).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,2-phenylene)diprop-2-yn-1-amide)-D-proline ([1,2]-19)**. Yield: 220 mg (0.38 mmol, 95%). ESI-MS *m/z* calcd for $C_{32}H_{42}N_4O_6 + H^+$: 579.4, obsd 579.4. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 – 7.31 (m, 2H), 7.23 – 7.16 (m, 2H), 6.67 (br s, 2H), 4.42 – 4.14 (m, 6H), 3.55 – 3.24 (br m, 4H), 2.29 – 1.75 (br m, 8H), 1.42 (s, 18H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.9, 154.3 (4 × C), 131.9, 128.5 (4 × CH), 125.6, 89.2, 81.3, 80.3 (8 × C), 60.9, 59.8 (2 × CH), 47.0 (2 × CH_2), 31.2, 29.8 (2 × CH_2), 29.5 (2 × CH_2), 28.3 (6 × CH_3), 24.6, 23.8 (2 × CH_2).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,3-phenylene)diprop-2-yn-1-amide)-D-proline ([1,3]-19)**. Yield: 175 mg (0.30 mmol, 76%). ESI-MS *m/z* calcd for $C_{32}H_{42}N_4O_6 + H^+$: 579.4, obsd 579.2. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 (s, 1H), 7.26 – 7.19 (m, 2H), 7.19 – 7.10 (m, 1H), 6.63 (br s, 2H), 4.42 – 4.13 (m, 6H), 3.55 – 3.26 (m, 4H), 2.29 – 2.01 (m, 3H), 2.00 – 1.79 (m, 5H), 1.46 (s, 18H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.8, 155.8 (4 × C), 134.6, 131.4, 128.1 (4 × CH Ar), 122.9, 85.7, 82.2, 80.5 (8 × C), 61.1, 59.8 (2 × CH), 47.1, 30.0, 29.7, 29.5 (6 × CH_2), 28.3 (6 × CH_3), 24.6, 23.8 (2 × CH_2).

***N,N'*-α,α'-Di-*tert*-Boc-(3,3'(1,3-phenylene)diprop-2-yn-1-amide)-D-proline ([1,4]-19)**. Yield: 172 mg (0.35 mmol, 88%). ESI-MS *m/z* calcd for $C_{32}H_{42}N_4O_6 + H^+$: 579.4, obsd 579.1. ^{1}H NMR (400 MHz, DMSO-*d*6) δ 7.30 (s, 4H), 6.65 (br s, 2H), 4.43 – 4.16 (m, 6H), 3.54 – 3.27 (m, 4H), 2.39 – 1.80 (m, 8H), 1.45 (s, 18H). ^{13}C NMR (100 MHz, DMSO-*d*6) δ 172.4, 153.2 (4 × C), 131.5 (4 × CH), 122.6, 86.9, 82.6, 80.5 (8 × C), 61.1, 59.9 (2 × CH_2), 47.1 (2 × CH_2), 31.0, 29.5 (2 × CH_2), 29.8 (2 × CH_2), 28.0 (6 × CH_3), 24.6, 23.8 (2 × CH_2).

Monomeric ligand [0,1]-D-pro-LHA. Boc protected compounds **[0,1]-19** (100 µmol, 32.8 mg) was subjected to a solution of DCM/TFA (4/1; v/v, 5 mL) for 18 h. The reaction mixture was concentrated and the residual solvents were removed by coevaporation with toluene. Accordingly, the crude free amine was dissolved in 100 µL of DMF and added to a solution containing pharmacophore **LHA-4** (100 µmol, 44.5 mg), BOP (150 µmol, 67.5 mg) and DiPEA (300 µmol, 51 µL) in 300 µL DMF. The reaction mixture was stirred at rt for 18 h and diluted with a mixture of DCM and MeOH (9/1; v/v, 20 mL). The organic layer was successively washed with water (3 × 10 mL), 10% aqueous $NaHCO_3$ (3 × 10 mL) and brine (20 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated. The crude product was purified on a semi-preparative HPLC system (40 to 60% B) and lyophilized from dioxane/H_2O to obtain **[0,1]-D-pro-LHA** as yellow amorphous solids. Yield after RP-HPLC purification: 8.2 mg (11 µmol, 11%). LC-MS analysis: *t*R 10.21 min (gradient 10 to 90% B). ESI-MS *m/z*: 656.2 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.43 – 7.37 (m, 2H), 7.31 – 7.26 (m, 3H), 7.22 – 7.16 (m, 1H), 7.14 – 7.11 (m, 1H), 6.90 (d, *J* = 7.4, 1H), 6.81 (d, *J* = 8.2, 1H), 6.72 (s, 1H), 6.06 (br s, 2H), 5.20 (s, 1H), 4.64 (d, *J* = 8.1, 1H), 4.25 (ddd, *J* = 5.2, 17.7, 22.3, 2H), 3.93 (s, 3H), 3.63 – 3.56 (m, 2H), 3.50 – 3.42 (m, 1H), 2.64 (s, 3H), 2.50 – 2.41 (m, 1H), 2.25 – 2.13 (m, 1H), 2.10 – 1.99 (m, 1H), 1.98 – 1.86 (m, 1H), 1.45 (s, 9H). HRMS *m/z* calcd for $C_{34}H_{37}N_7O_3S_2 + H^+$: 656.24721, obsd 656.24704.

***General procedure for coupling of LHA-4 with dimeric spacers [1,2]-19, [1,3]-19 or [1,4]-19 affording* [1,2]-D-pro-LHA, [1,3]-D-pro-LHA and [1,4]-D-pro-LHA.**

Boc protected compounds **[1,2]-19**, **[1,3]-19** or **[1,4]-10** (50 µmol, 28.9 mg) were subjected to a solution of 4/1 DCM/TFA v/v (5 mL) for 18 h. The reaction mixture was concentrated and the residual solvents were removed by coevaporation with toluene. Accordingly, the free amine was dissolved in 100 µL of DMF and added to a solution containing pharmacophore **LHA-4** (100 µmol, 44.5 mg), BOP (150 µmol, 67.5 mg) and DiPEA (300 µmol, 51 µL) in 300 µL DMF. The reaction mixture was stirred at rt for 18 h and diluted with a mixture of DCM and MeOH (9/1; v/v, 20 mL). The organic layer was successively washed with water (3 × 10 mL), 10% aqueous $NaHCO_3$ (3 × 10 mL) and brine (20 mL). The organic layer was dried (Na_2SO_4), filtered and concentrated. The crude product was purified on a semi-preparative HPLC system (40 to 60% B) and lyophilized from dioxane/H_2O to obtain **[1,2]-D-pro-LHA**, **[1,3]-D-pro-LHA** and **[1,4]-D-pro-LHA** as yellow amorphous solids.

[1,2]-D-pro-LHA. Yield after RP-HPLC purification: 3.8 mg (2.6 µmol, 5%). LC-MS analysis: *t*R 12.1 min (gradient 30 to 90% B). ESI-MS *m/z*: 1233.4 $[M + H]^+$. ^1H NMR (400 MHz, $CDCl_3$) δ 7.37 – 7.28 (m, 4H), 7.32 – 7.28 (m, 2H), 6.87 (d, *J* = 7.4, 2H), 6.80 (d, *J* = 7.8, 2H), 6.65 (s, 2H), 6.07 (br s, 4H), 5.20 (br s, 2H), 4.50 (d, *J* = 6.6, 2H), 4.33 – 4.20 (m, 4H), 3.91 (s, 4H), 3.64 – 3.53 (m, 2H), 3.49 – 3.38 (m, 2H), 2.62 (s, 6H), 2.27 – 2.10 (m, 4H), 2.04 – 1.90 (m, 4H), 1.43 (s, 18H). HRMS *m/z* calcd for $C_{62}H_{68}N_{14}O_6S_4 + H^+$: 1233.44019, obsd 1233.44194.

[1,3]-D-pro-LHA. Yield after RP-HPLC purification: 2.2 mg (1.5 µmol, 3%). LC-MS analysis: *t*R 11.5 min (gradient 30 to 90% B). ESI-MS *m/z*: 1233.4 $[M + H]^+$. ^1H NMR (400 MHz, CDCl3) δ 7.83 – 7.69 (m, 2H), 7.37 – 7.24 (m, 6H), 6.89 (d, *J* = 7.4, 2H), 6.80 (d, *J* = 8.1, 2H), 6.73 (s, 2H), 6.05 (br s, 4H), 5.21 (br s, 2H), 5.00 (br s, 2H), 4.65 – 4.59 (m, 2H), 4.23 (ddd, *J* = 5.4, 17.7, 22.5, 4H), 3.92 (s, 4H), 3.63 – 3.53 (m, 2H), 3.50 – 3.40 (m, 2H), 2.63 (s, 6H), 2.50 – 2.41 (m, 2H), 2.25 – 2.12 (m, 2H), 2.09 – 1.98 (m, 2H), 2.97 – 1.85 (m, 2H), 1.44 (s, 18H). HRMS *m/z* calcd for $C_{62}H_{68}N_{14}O_6S_4 + H^+$:1233.44019, obsd 1233.44131.

[1,4]-D-pro-LHA. Yield after RP-HPLC purification: 5.6 mg (3.4 µmol, 8%). LC-MS analysis: *t*R 11.5 min (gradient 30 to 90% B). ESI-MS *m/z*: 1233.4 $[M + H]^+$. ^1H NMR (400 MHz, CDCl3) δ 7.38 – 7.25 (m, 6H), 6.89 (d, *J* = 7.1, 2H), 6.80 (d, *J* = 8.1, 2H), 6.72 (s, 2H), 6.06 (br s, 4H), 5.22 (br s, 2H), 4.99 (br s, 2H), 4.63 (d, *J* = 6.7, 2H), 4.25 (ddd, *J* = 5.2, 17.6, 59.7, 4H), 3.92 (s, 4H), 3.63 – 3.55 (m, 2H), 3.51 – 3.42 (m, 2H), 2.64 (s, 6H), 2.50 – 2.41 (m, 2H), 2.27 – 2.13 (m, 2H), 2.09 – 1.99 (m, 2H), 1.98 – 1.86 (m, 2H), 1.44 (s, 18H). HRMS *m/z* calcd for $C_{62}H_{68}N_{14}O_6S_4 + H^+$: 1233.44019, obsd 1233.44168.

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Chapter 7

Oligoproline helix as a scaffold for potent, selective and structurally defined dimeric ligands for the LHR

Introduction

The luteinizing hormone receptor (LHR)[1] and follicle-stimulating hormone receptor (FSHR)[2] play an important role in human reproduction. They belong to the family of G-protein coupled receptors (GPCRs) that are characterized by a seven helical transmembrane region. The LHR and FSHR are activated by binding of the endogenous glycoprotein ligand to the large N-terminal leucine rich repeat (LRR).[3,4] Upon activation by LH in male Leydig cells, LHR induces testosterone production. In females, LH is primarily responsible for ovulation induction and FSH induces follicular growth in the ovaries. In males, FSH is involved in spermatogenesis.
Recent developments in fertility treatment led to the discovery of several low molecular weight (LMW) agonists for the LH receptor of which some also activate the FSH receptor.[5-8] Chapter 5 described that increased selectivity for the LHR can be realized by dimerization of a known LMW LHR agonist (LHA). The dimeric ligands were based on both flexible polyethylene glycol spacers and a more rigid benzene substituted core. Since the spatial orientation of such spacer systems is difficult to establish, it was not possible to explain the observed selectivity increase by speculation

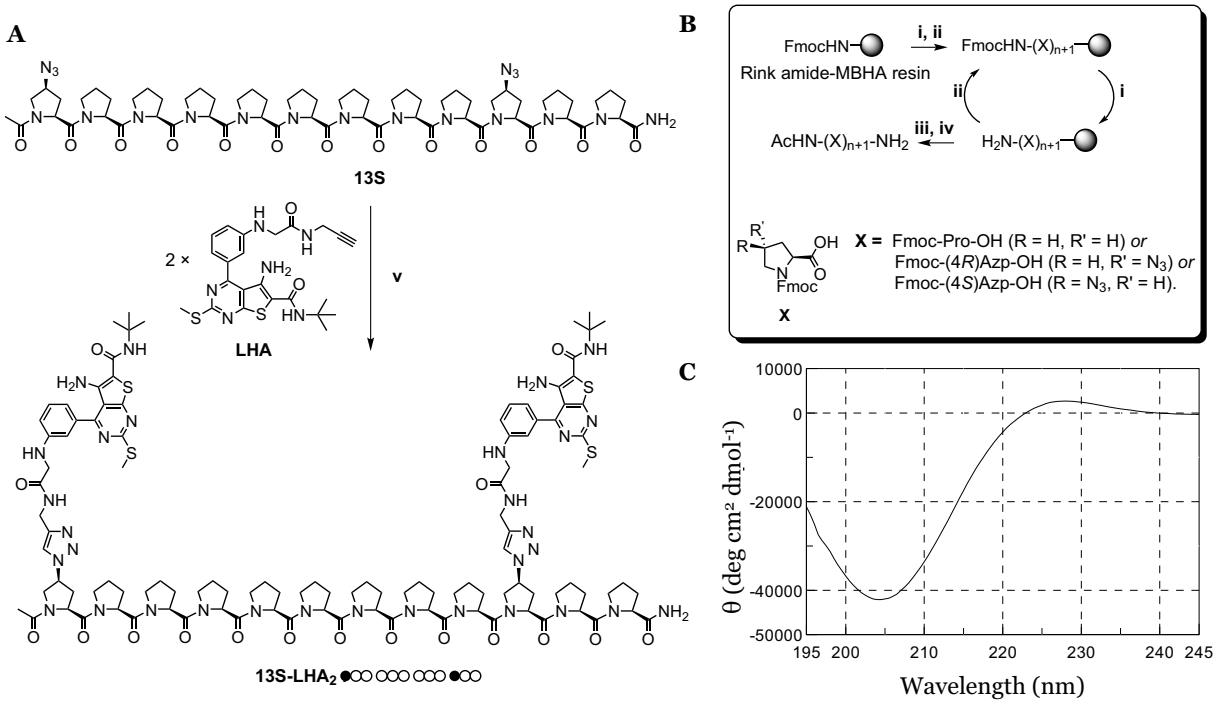
on the interpharmacophoric distance or binding mode of the dimeric ligands to the receptors. It was reasoned that scaffolds based on a defined tertiary structure may help in elucidating how dimeric ligands act on GPCRs.

Oligoprolines (OP), composed of at least three proline residues, adopt well-defined helical structures.[9-11] Proline helices are common in biological systems and play a part in many protein structures and protein-protein interactions.[12-14] In (bio)physical chemistry, oligoproline helices are widely used as molecular rulers to calibrate distances in resonance energy transfer experiments[15,16] or as backbones to obtain amphiphilic molecules after decoration with specific side-chains.[17] Remarkably, only a few reports applied the proline helices as a scaffold or spacer system for interconnecting two or more biologically active molecules.[18-20] This chapter describes the use of the proline helix as a well defined, water soluble scaffold in the preparation of dimeric ligands for the LHR.

Results and discussion

The first objective was to prepare a set of oligoprolines containing several 4-azidoproline (Azp) residues for ensuing installation of the LHR agonist, by means of a Huisgen [2+3]-cycloaddition reaction. The OPs were designed to vary in length, the position and number of the Azp-residues incorporated. Both (4*R*)- and (4*S*)-azidoproline (Azp) were prepared as described.[21,22] The helices were constructed by solid phase peptide synthesis (SPPS) as depicted in Scheme 1. The OPs were subsequently cleaved from the resin and subjected to a Huisgen [2+3]-cycloaddition with the LHR agonist (LHA) that was prepared as described in chapter 5. A representative example of the synthesis of dimeric ligand **13S-LHA$_2$** is depicted in Scheme 2. The reactions ran to completion in three hours at 60°C with one equivalent of copper sulfate, five equivalents of sodium ascorbate in a water/*tert*-butanol/acetonitrile mixture (Scheme 2). The end products and substitution patterns are listed in Table 1.

The compounds were assayed for their potency to activate both the LHR and FSHR. As is shown in Table 1, all compounds are potent LHR agonists, whereas their FHSR agonistic potency is at most rather modest. Some general trends are observed within the series. For example, compounds that are substituted with two LHAs are 2-5 times more potent on the LHR than the compounds with only one LHA. The most potent LHR agonist, **19R-LHA$_3$** incorporating three LHAs, is at least 7 times more potent than the compounds with one LHA and about four times more potent on the LHR than those with two LHAs. Compounds **20-LHA$_4$**, containing four LHAs, are less potent than **19-LHA$_3$**. A similar increase in potency is also observed for the FSHR which increases with the number of LHA ligands. For this receptor, the most potent compounds are **19LHA$_3$**, as was also observed for the LHR.



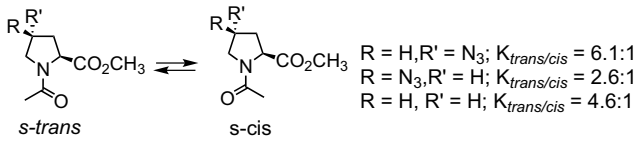
Scheme 1. A. Representative example for the preparation of ligands containing LHR agonist (**LHA**) by the copper catalyzed [2+3]-Huisgen cycloaddition. ○ represents proline; ● represents LHA functionalized proline. B. synthetic scheme of OP scaffolds. *Reagents and conditions*: *i*. 20% piperidine/DMF, 0.5h; *ii*. Fmoc-Pro-OH or Fmoc-Azp-OH, HCTU, DiPEA, NMP, 3 h; *iii*. Ac_2O, DiPEA, DMF, 2 h; *iv*. 95% TFA/H_2O, 1h; *v*. Sodium ascorbate (5 eq), $CuSO_4$ (1 eq), *t*BuOH/CH_3CN/H_2O. C. CD-spectra of compound **13S-LHA$_2$**. The intensity in ellipticity observed for **13S-LHA$_2$** ($4E^{-5}$ M in 10% *i*PrOH/phosphate buffer pH 7.2) shows a minimum at 205 nm and maximum at 228 nm.

There are some small differences observed in the bioactivity between the *R*-series and the *S*-series (compare Table 1 left panel with Table 1 right panel). The mono-substituted compounds bearing the 4*R* substituted proline are slightly more potent on the LHR and the FSHR when compared to the compounds with 4*S* substituted prolines. This is especially true when the ligand is attached close to the N- or C-terminus of the helix (that is for compounds **1-LHA** and **4-LHA**). The potencies for the compounds that contain two agonists are in the same order of magnitude for both the LHR and the FSHR and do not differ dramatically between the *R* and *S*-series. Compounds **14R-LHA2** and **14S-LHA2** are the least potent for both the receptors compared to the other dimeric ligands. For these compounds, both the LHA ligands are attached in the middle of the helix. Compounds with more than two ligands (that is, **19-LHA$_3$** and **20-LHA$_4$**) have similar potency between the *R*- and *S*-series for the LHR. For the FSHR, the compounds with *R*-substituted ligands are slightly more potent than the compounds in where the ligands are *S*-substituted.

	EC_{50} (nM)			*Compound*		EC_{50} (nM)		
	FSHR	**LHR**	**FSHR/ LHR[a]**			**FSHR**	**LHR**	**FSHR/ LHR[a]**
				Monomeric				
1R-LHA	2109	32	67	●○○ ○○○ ○○○ ○○○	**1S-LHA**	>3000	76	40
2R-LHA	>3000	98	>31	○○○ ●○○ ○○○ ○○○	**2S-LHA**	>3000	110	>27
3R-LHA	>3000	107	>28	○○○ ○○○ ●○○ ○○○	**3S-LHA**	>3000	114	>26
4R-LHA	>3000	44	>69	○○○ ○○○ ○○○ ●○○	**4S-LHA**	>3000	84	>36
				Dimeric				
5R-LHA$_2$	1333	20	67	●●○ ○○○ ○○○ ○○○	**5S-LHA$_2$**	2092	26	80
6R-LHA$_2$	1427	20	71	●○● ○○○ ○○○ ○○○	**6S-LHA$_2$**	2362	32	75
7R-LHA$_2$	1499	27	56	●○○ ●○○ ○○○ ○○○	**7S-LHA$_2$**	2367	30	80
8R-LHA$_2$	1442	23	63	●○○ ○●○ ○○○ ○○○	**8S-LHA$_2$**	1007	15	66
9R-LHA$_2$	1696	28	60	●○○ ○○● ○○○ ○○○	**9S-LHA$_2$**	2042	27	75
10R-LHA$_2$	1498	29	51	●○○ ○○○ ●○○ ○○○	**10S-LHA$_2$**	1898	27	71
11R-LHA$_2$	1493	27	54	●○○ ○○○ ○●○ ○○○	**11S-LHA$_2$**	1284	22	59
12R-LHA$_2$	1704	27	62	●○○ ○○○ ○○● ○○○	**12S-LHA$_2$**	1266	21	59
13R-LHA$_2$	1335	25	54	●○○ ○○○ ○○○ ●○○	**13S-LHA$_2$**	1178	20	60
14R-LHA$_2$	2267	33	68	○○○ ●○○ ●○○ ○○○	**14S-LHA$_2$**	2707	32	84
15R-LHA$_2$	1999	31	65	○○○ ○○○ ●○○ ●○○	**15S-LHA$_2$**	1200	33	36
16R-LHA$_2$	1223	16	76	●○○ ○○○ ○○○ ○○○ ●○○	**16S-LHA$_2$**	1499	21	73
17R-LHA$_2$	2193	27	82	●○○ ○○○ ○○○ ○○○ ○○○ ●○○	**17S-LHA$_2$**	1766	25	71
18R-LHA$_2$	1518	18	84	●○○ ○○○ ○○○ ○○○ ○○○ ○○○ ●○○	**18S-LHA$_2$**	2066	28	73
				Tri- and Tetrameric				
19R-LHA$_3$	114	5	25	●○○ ●○○ ●○○ ○○○	**19S-LHA$_3$**	331	5	70
20R-LHA$_4$	1067	9	65	●○○ ●○○ ●○○ ●○○	**20S-LHA$_4$**	1764	9	188

Table 1. Mean agonistic potency (EC_{50}) and selectivity for the LHR and FSHR. All compounds are full agonists on the LHR and partial agonists on the FSHR. The mean EC_{50} are calculated from the -log EC_{50} values from two or three independent experiments performed in duplicate. The SD of pEC_{50} is generally lower than 0.2. ○ represents proline; ● represents LHA functionalized proline. [a] Selectivity observed for the LHR (EC_{50} FSHR/EC_{50} LHR).

The observed differences in bioactivity within the (*R*)- and (*S*)-substituted prolines may be attributed to the relative stability of the proline helix. In literature, two types of polyproline helices are distinguished that differ in the configuration of the proline interconnecting amide bonds.[9-11] PP type I (PPI) is defined as the structure resulting from *cis* interconnecting amide bonds. These α-helical structures are characterized by 3.3 proline residues per turn with a rise of 1.9 Å per residue. PP type II helices (PPII) assemble from all *trans* amide bonds, are more common and feature three proline residues in one turn. The PPII helix is less dense than PPI and the rise of one residue is approximately 3.1 Å. Wennemers *et al.* demonstrated that functionalization of proline with an (4*R*)-azide stabilizes the *trans*-amide isomer by n→π* interaction of the carbonyl



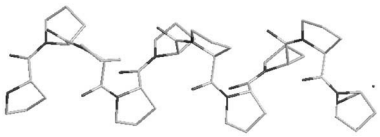
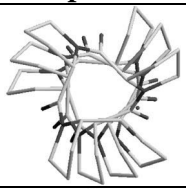
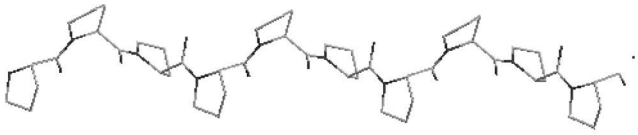
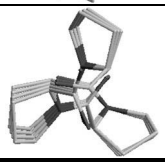
	Side-view	Top-view
PP type I:		
PP type II:		

Figure 1. s-*Trans*/s-*cis* isomer ratios for different substituted prolines. Below: Side and top-view of polyproline type I and II helix.

functions while (4*S*)-azidoproline (Azp) directs the equilibrium towards the *cis*-isomer.[23,24] This effect was also observed when other electron-withdrawing substituents, such as fluorine, were incorporated at the 4-position (Figure 1).[25-27]

The helical distribution of the compounds can be evidenced by circular dichroism (CD) experiments. PPI type helices are characterized by a minimum at 230 nm and a maximum at 212 nm in their CD spectra, while PPII type helix has maximum at 225 nm and a minimum at 207 nm. All compounds, both the ligand-functionalized derivatives from Table 1 and their azidoproline containing precursors, were evaluated by circular dichroism experiments. In all cases a spectrum indicative for PP type II helix was observed, independent of the spacer length and substitution pattern when measured in a 10% *i*PrOH/phosphate buffer (minimum at 205 nm, maximum at 228 nm). It is generally observed that compounds with more stable PP type II helices have more intense ellipticities at 205 nm than compounds that equilibrate faster with PP type I configuration.[9,23] Compounds **5-13** all possess polyproline helices of similar lengths. Within these series, a more intense ellipticity of the (4*R*)-azidoproline containing helices was observed, compared to the corresponding (4*S*)-Azp containing helices (Figure 2, left). This is in agreement with the results described by Wennemers et. al.[23] The reverse trend is observed when the helices are substituted with a HLA ligand. Here, the (4*R*)-LHAs have less intense ellipticities than the (4*S*)-LHAs (Figure 3, right). This may indicate that the triazole substituted proline may destabilize the PPII conformation.[28,29]

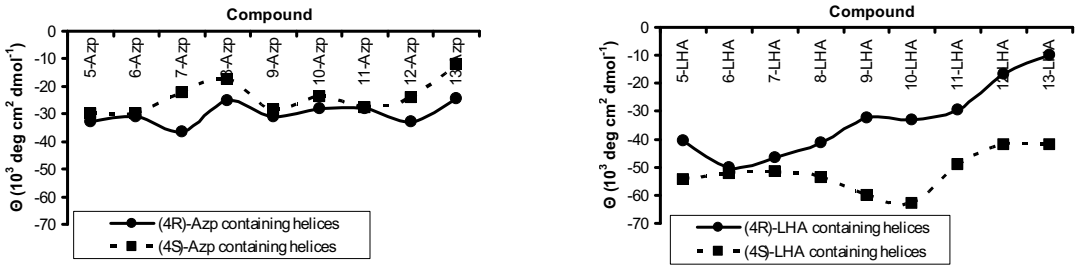


Figure 2. Ellipticity of PP type II helix of dimeric azidoproline containing compounds **5-13-Azp** and LHA functionalized proline compounds **5-13-LHA**. The intensity in ellipticity observed for samples ($4E^{-5}$ M in 10% *i*PrOH/phosphate buffer pH 7.2) at 205 nm is depicted.

Previous studies towards the bioactivity of dimeric LHA ligands described in Chapter 5, indicated that, upon dimerization, improved selectivity towards the LHR was observed compared to the monomeric ligands. This was either a result of reduced potency or a reduced efficacy on the FSHR upon dimerization of the LHA ligands. In the here presented series, all ligands are full LHR agonists and partial FSHR agonists (E_{max} between 40 and 72). Only compound **20S-LHA$_4$**, with four LHA ligands, shows an increase in selectivity compared to the other compounds. The reduced FSHR efficacy of dimeric compounds in comparison with the monomeric compounds was not observed when the oligoproline spacer was used.

Conclusion

In summary, this chapter describes the use of a polyproline type helix as a scaffold for interconnecting multiple LHAs. For all synthesized compounds a typical PP type II helix was evidenced by circular dichroism indicating that decoration of the helix with large LHR agonists did not affect the helical conformation. LMW LHAs not only activate the LHR but generally also trigger the FSHR. Pharmacological evaluation revealed two interesting features of the oligomerization of LHR agonists with the use of this scaffold. 1) A significant increase in potency on the LHR that is related to the increase in LHA functionalized prolines on the helix. 2) An increase in selectivity for the LHR compared to FSHR for compound **20S-LHA$_4$**, that holds four LHA ligands. These features indicate that oligoproline is a suitable scaffold for the development of dimeric or oligomeric ligands as probes to study the dimeric ligand effect to G-protein coupled receptors in more detail.

Experimental procedures

Measurement of CRE-induced luciferase activity

Materials. Recombinant human LH (recLH) and human recombinant FSH (recFSH) were synthesized at Schering-Plough Research Institute, Oss, The Netherlands. Luclite® was obtained from Packard. All cell culture supplies were obtained from Gibco/BRL unless indicated otherwise. The human LH receptor cDNA[30] and human FSH receptor cDNA[31] were kindly provided by Dr. A.J.W. Hsueh, Stanford University.

Luciferase assay. Chinese Hamster Ovary (CHO)-K1 cells stably expressing the CRE-luciferase reporter with the human LH receptor or human FSH receptor were grown to 80-90% confluency in Dulbecco's MEM/Nutrient Mix F12 containing 5% bovine calf serum and supplemented with penicillin G (80 units/mL) and streptomycin (0.08 mg/mL) in 5% CO_2 at 37 °C. Cells were harvested using cell dissociation solution (Sigma). Aliquots of the cells were cryopreserved in DMSO without a loss of functional activity on LH receptor or FSH receptor.[32] On the day of the experiment, cells were thawed, washed with assay medium (Dulbecco's MEM/Nutrient Mix F12 supplemented with 1 mg/L bovine insulin (Sigma), 5 mg/L apo-transferrin (Sigma), penicillin G (80 units/mL) and streptomycin (0.08 mg/mL)) and then resuspended in assay medium. The compounds were tested at 10 concentrations ranging from final concentrations of 10 μM to 0.316 nM with half log intervals. In the agonistic assays, 10 μL of assay medium containing test compound and 3% DMSO, 10 μL of assay medium containing 3% DMSO with recLH (final concentration of 1 nM) or recFSH (final concentration of 586 pM) or 10 μL of assay medium containing 3% DMSO alone were added to the wells of a 384-well white culture plate followed by the addition of 10 μL of assay medium. Then, 10 μL of cell suspension containing 7,500 cells was added to the wells. The final concentration of DMSO was 1%. After incubation for 4 h in a humidified atmosphere in 5% CO_2 at 37 °C, plates were allowed to adjust to room temperature for 1 h. Then, 15 μL of LucLite solution (Packard) was added to the incubation mixture. Following 60 min at room temperature in the dark, luciferase activity was measured in a Packard Topcount Microplate Scintillation and Luminescence Counter. Agonistic effects of the compounds were determined as percentage of the (maximal) effect induced by 1 nM recLH or 586 pM recFSH. The EC_{50} values (concentration of the test compound that elicits half-maximal (50%) luciferase stimulation compared to the compound's maximally attainable effect, respectively) and the efficacy values (maximal effect as percentage of the effect of recLH or recFSH) of the test compounds were determined using the software program MathIQ (version 2.0, ID Business Solutions Limited).

Chemical procedures

NMR spectra were recorded on a 400/100 MHz, 500/125 MHz or 600/150 MHz spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants (*J*) are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. Where indicated, NMR peak assignments were made using COSY, NOESY (τ mix = 1 sec) and HMQC experiments. For LC-MS analysis, a HPLC-system (detection simultaneously at 214 and 254 nm) equipped with an analytical C_{18} column (4.6 mmD x 250 mmL, 5μ particle size) in combination with buffers A: H_2O, B: CH_3CN and C: 1% aq TFA and coupled to a mass instrument with an electronspray interface (ESI) was used. For RP-HPLC purifications, an automated HPLC system equipped with a semi-preparative C_{18} column (5 μm C_{18}, 10Å, 150 x 21.2 mm) was used. The applied buffers were A: H_2O + ammonium acetate (20 mM) and B: CH_3CN. High resolution mass spectra were recorded by direct injection (2 μL of a 2 μM solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60000 at *m/z* 400 (mass range *m/z* = 150-2000) and dioctylpthalate (*m/z* = 391.28428) as a lock mass. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). CD spectra were recorded using a spectral

bandwidth of 1 nm, at 25 °C with a response time of 2 s. The spectra are the result of 2-3 accumulations. A peptide solution was measured in a concentration of 4E-5 M in 10% *i*-PrOH/phosphate buffer (10 mM, pH 7.2) in a quartz cell of 2 mm. CD data is given as mean residual molar ellipticities (θ in deg cm^2 $dmol^{-1}$). All samples were equilibrated at least 12 h before measurement.

General procedure for SPPS of azido-containing polyprolines.
Rink amide MBHA resin (loading 0.64 mmol/g, 78 mg, 0.05 mmol) was preswollen in DMF for 30 min, drained and the Fmoc protecting group removed with 20% piperidine in NMP. After shaking for 30 min, the resin was drained, washed with NMP (3×), DCM (5×) and NMP (3×). Subsequently, Fmoc-Pro-OH or Fmoc-(*R/S*)Azp-OH (3 eq) and HCTU (3 eq) are dissolved in NMP followed by DiPEA (9 eq). After standing for 5 min the mixture was added to the amino-functionalized resin (preswollen in NMP). After shaking for 3 h, the resin was drained, washed with NMP (3×), DCM (5×) and NMP (3×). Acetylation was accomplished by adding Ac_2O (5 eq) and DiPEA (5 eq) in DMF to the resin and shaken for 2 h. The resin was drained, washed with NMP (3×), DCM (5×) and NMP (3×). All couplings were monitored by the qualitative Chloranil test[33] The polyproline peptide was cleaved from the resin by stirring in 95% TFA/H_2O (2 mL) for 2 h. The solution was then titrated in 40 mL of Et_2O and centrifuged. The solvent was decanted and the polyproline peptide dissolved in H_2O and purified by preparative HPLC (0 to 20 % B) to yield the compounds as white solids.

Monomeric azidoproline helix 1R-Azp. Yield after RP-HPLC purification: 16.0 mg (12.6 µmol, 25%). LC-MS analysis: t_R 5.07 min (gradient 10 to 50% B). ESI-MS *m/z*: 1265.7 $[M + H]^+$. ^1H NMR (400 MHz, D_2O) δ 4.79 – 4.72 (m, 11H, Hα), 4.54 – 4.48 (m, 1H, Hγ-Azp), 4.42 (dd, *J* = 5.5, 8.4, 1H, Hα-Pro-NH_2), 3.94 – 3.82 (m, 12H, Hδ-Azp, Hδ), 3.74 – 3.59 (m, 12H, Hδ'), 2.57 (ddd, *J* = 1.5, 7.6, 11.4, 1H, Hβ-Azp), 2.44 – 2.30 (m, 11H, Hβ), 2.15 (s, 3H, CH_3), 2.23 – 2.03 (m, 24H, 1 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 2.01 – 1.92 (m, 10H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{68}N_{16}O_{13}$ + H^+: 1265.67895, obsd 1265.67892.

Monomeric azidoproline helix 2R-Azp. Yield after RP-HPLC purification: 37.8 mg (30.0 µmol, 60%). LC-MS analysis: t_R 5.05 min (gradient 10 to 50% B). ESI-MS *m/z*: 1265.7 $[M + H]^+$. ^1H NMR (400 MHz, D_2O) δ 4.78 – 4.70 (m, 11H, Hα), 4.59 – 4.52 (m, 1H, Hγ-Azp), 4.41 (dd, *J* = 5.5, 8.4, 1H, Hα-Pro-NH_2), 3.99 (d, *J* = 12.2, 1H, Hδ-Azp), 3.93 – 3.80 (m, 11H, Hδ), 3.74 – 3.52 (m, 12H, Hδ'), 2.53 (ddd, *J* = 2.5, 8.3, 12.2, 1H, Hβ-Azp), 2.44 – 2.29 (m, 11H, Hβ), 2.13 (s, 3H, CH_3), 2.18 – 2.01 (m, 24H, 1 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 2.02 – 1.87 (m, 10H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{68}N_{16}O_{13}$ + H^+: 1265.67895, obsd 1265.67897.

Monomeric azidoproline helix 3R-Azp. Yield after RP-HPLC purification: 43.8 mg (34.6 µmol, 69%). LC-MS analysis: t_R 5.00 min (gradient 10 to 50% B). ESI-MS *m/z*: 1265.7 $[M + H]^+$. ^1H NMR (400 MHz, D_2O) δ 4.88 – 4.69 (m, 11H, Hα), 4.59 – 4.51 (m, 1H, Hγ-Azp), 4.41 (dd, *J* = 5.3, 8.4, 1H, Hα-Pro-NH_2), 3.99 (d, *J* = 11.6, 1H, Hδ-Azp), 3.94 – 3.81 (m, 11H, Hδ), 3.74 – 3.65 (m, 12H, Hδ'), 2.53 (ddd, *J* = 1.5, 7.2, 10.2, 1H, Hβ-Azp), 2.45 – 2.28 (m, 11H, Hβ), 2.13 (s, 3H, CH_3), 2.12 – 2.01 (m, 24H, 1 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 2.01 – 1.86 (m, 10H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{68}N_{16}O_{13}$ + H^+: 1265.67895, obsd 1265.67870.

Monomeric azidoproline helix 4R-Azp. Yield after RP-HPLC purification: 8.7 mg (6.9 µmol, 14%). LC-MS analysis: t_R 4.94 min (gradient 10 to 50% B). ESI-MS *m/z*: 1265.7 $[M + H]^+$. ^1H NMR (400 MHz, D_2O) δ 4.91 – 4.68 (m, 11H, Hα), 4.60 – 4.52 (m, 1H, Hγ-Azp), 4.41 (dd, *J* = 5.4, 8.4, 1H, Hα-Pro-NH_2), 3.99 (d, *J* = 11.1, 1H, Hδ-Azp), 3.95 – 3.80 (m, 11H, Hδ), 3.73 – 3.52 (m, 12H, Hδ'), 2.54 (ddd, *J* = 3.1, 7.7, 10.4, 1H, Hβ-Azp), 2.46 – 2.26 (m, 11H, Hβ), 2.13 (s, 3H, CH_3), 2.19 – 2.02 (m, 24H, 1 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 2.02 – 1.84 (m, 10H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{68}N_{16}O_{13}$ + H^+: 1265.67895, obsd 1265.67898.

Dimeric azidoproline helix 5R-Azp$_2$. Yield after RP-HPLC purification: 33.2 mg (25.5 μmol, 51%). LC-MS analysis: t_R 5.56 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.8 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.82 (t, *J* = 8.1, 1H, Hα-Azp), 4.75 (t, *J* = 8.0, 1H, Hα-Azp), 4.72 – 4.66 (m, 9H, Hα), 4.45 (ddd, *J* = 3.0, 5.2, 7.5, 1H, Hγ-Azp), 4.46 (ddd, *J* = 2.9, 5.1, 7.8, 1H, Hγ-Azp), 4.35 (dd, *J* = 5.5, 8.6, 1H, Hα-Pro-NH_2), 4.02 (d, *J* = 12.3, 1H, Hδ-Azp), 3.86 – 3.76 (m, 12H, Hδ), 3.71 (d, *J* = 12.1, 1H, Hδ'), 3.65 – 3.56 (m, 10H, Hδ'), 2.51 – 2.44 (m, 2H, Hβ-Azp), 2.36 – 2.26 (m, 9H, Hβ), 2.13 – 2.07 (m, 2H, Hβ'-Azp), 2.09 (s, 3H, CH_3), 2.05 – 1.98 (m, 21H, 1 × Hβ-Pro-NH_2, Hγ), 1.92 – 1.85 (m, 10H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68115.

Dimeric azidoproline helix 6R-Azp$_2$. Yield after RP-HPLC purification: 31.5 mg (24.1 μmol, 48%). LC-MS analysis: t_R 5.48 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.77 (t, *J* = 8.0, 1H, Hα-Azp), 4.74 – 4.67 (m, 10H, Hα), 4.49 (ddd, *J* = 2.8, 5.1, 7.6, 1H, Hγ-Azp), 4.46 (ddd, *J* = 2.8, 5.2, 7.8, 1H, Hγ-Azp), 4.35 (dd, *J* = 5.5, 8.6, 1H, Hα-Pro-NH_2), 3.93 (d, *J* = 12.3, 1H, Hδ-Azp), 3.86 – 3.78 (m, 12H, Hδ), 3.72 (d, *J* = 12.1, 1H, Hδ'), 3.66 – 3.57 (m, 10H, Hδ'), 2.54 – 2.47 (m, 2H, Hβ-Azp), 2.36 – 2.25 (m, 9H, Hβ), 2.15 – 2.09 (m, 2H, Hβ'-Azp), 2.09 (s, 3H, CH_3), 2.06 – 1.99 (m, 21H, 1 × Hβ-Pro-NH_2, Hγ), 1.95 – 1.86 (m, 10H, 1 × Hβ'Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68115.

Dimeric azidoproline helix 7R-Azp$_2$. Yield after RP-HPLC purification: 37.5 mg (28.7 μmol, 57%). LC-MS analysis: t_R 5.70 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.77 (t, *J* = 7.6, 1H, Hα-Azp), 4.73 – 4.66 (m, 10H, Hα), 4.49 (ddd, *J* = 3.0, 5.3, 7.6, 1H, Hγ-Azp), 4.44 (ddd, *J* = 2.9, 5.3, 7.9, 1H, Hγ-Azp), 4.35 (dd, *J* = 5.5, 8.6, 1H, Hα-Pro-NH_2), 3.93 (d, *J* = 11.4, 1H, Hδ-Azp), 3.87 – 3.76 (m, 12H, Hδ), 3.71 (d, *J* = 11.8, 1H, Hδ'), 3.66 – 3.56 (m, 10H, Hδ'), 2.53 – 2.45 (m, 2H, Hβ-Azp), 2.36 – 2.24 (m, 9H, Hβ), 2.16 – 2.06 (m, 2H, Hβ'-Azp), 2.09 (s, 3H, CH_3), 2.06 – 1.97 (m, 21H, 1 × Hβ-Pro-NH_2, Hγ), 1.94 – 1.84 (m, 10H, 1 × Hβ'Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68164.

Dimeric azidoproline helix 8R-Azp$_2$. Yield after RP-HPLC purification: 18.8 mg (14.4 μmol, 29%). LC-MS analysis: t_R 5.61 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.77 (t, *J* = 8.0, 1H, Hα-Azp), 4.75 – 4.68 (m, 10H, Hα), 4.50 (ddd, *J* = 3.5, 5.8, 7.7, 1H, Hγ-Azp), 4.46 (ddd, *J* = 3.2, 5.3, 7.7, 1H, Hγ-Azp), 4.36 (dd, *J* = 5.5, 8.6, 1H, Hα-Pro-NH_2), 3.94 (d, *J* = 11.0, 1H, Hδ-Azp), 3.88 – 3.78 (m, 12H, Hδ), 3.72 (d, *J* = 11.6, 1H, Hδ'), 3.67 – 3.57 (m, 10H, Hδ'), 2.55 – 2.46 (m, 2H, Hβ-Azp), 2.37 – 2.26 (m, 9H, Hβ), 2.16 – 2.08 (m, 2H, Hβ'-Azp), 2.10 (s, 3H, CH_3), 2.06 – 1.96 (m, 21H, 1 × Hβ-Pro-NH_2, Hγ), 1.95 – 1.86 (m, 10H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68152.

Dimeric azidoproline helix 9R-Azp$_2$. Yield after RP-HPLC purification: 30.4 mg (23.3 μmol, 47%). LC-MS analysis: t_R 5.43 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.9 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.77 (t, *J* = 8.3, 1H, Hα-Azp), 4.74 – 4.67 (m, 10H, Hα), 4.49 (ddd, *J* = 3.0, 5.3, 7.9, 1H, Hγ-Azp), 4.46 (ddd, *J* = 3.1, 5.3, 7.8, 1H, Hγ-Azp), 4.35 (dd, *J* = 5.5, 8.6, 1H, Hα-Pro-NH_2), 3.93 (d, *J* = 12.3, 1H, Hδ-Azp), 3.87 – 3.77 (m, 12H, Hδ), 3.71 (d, *J* = 11.4, 1H, Hδ'), 3.67 – 3.56 (m, 10H, Hδ'), 2.54 – 2.45 (m, 2H, Hβ-Azp), 2.37 – 2.25 (m, 9H, Hβ), 2.15 – 2.07 (m, 2H, Hβ'-Azp), 2.09 (s, 3H, CH_3), 2.06 – 1.96 (m, 21H, 1 × Hβ-Pro-NH_2, Hγ), 1.95 – 1.84 (m, 10H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68127.

Dimeric azidoproline helix 10R-Azp$_2$. Yield after RP-HPLC purification: 26.2 mg (20.1 μmol, 40%). LC-MS analysis: t_R 5.59 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.77 (t, *J* = 8.0, 1H, Hα-Azp), 4.74 – 4.68 (m, 10H, Hα), 4.50 (ddd, *J* = 3.0, 5.3, 7.8, 1H, Hγ-Azp), 4.46 (ddd, *J* = 3.1, 5.3, 8.0, 1H, Hγ-Azp), 4.36 (dd, *J* = 5.5, 8.6, 1H, Hα-Pro-NH_2), 3.94 (d, *J* = 12.1, 1H, Hδ-Azp), 3.87 – 3.78 (m, 12H,

Hδ), 3.72 (d, *J* = 12.1, 1H, Hδ'), 3.66 – 3.57 (m, 10H, Hδ'), 2.54 – 2.46 (m, 2H, Hβ-Azp), 2.37 – 2.26 (m, 9H, Hβ), 2.16 – 2.07 (m, 2H, Hβ'-Azp), 2.09 (s, 3H, CH_3), 2.07 – 1.98 (m, 21H, 1 × Hβ-Pro-NH_2, Hγ), 1.95 – 1.85 (m, 10H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68140.

Dimeric azidoproline helix 11R-Azp$_2$. Yield after RP-HPLC purification: 46.4 mg (35.6 μmol, 71%). LC-MS analysis: t_R 5.59 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.8 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.77 (t, *J* = 8.0, 1H, Hα-Azp), 4.74 – 4.68 (m, 10H, Hα), 4.50 (ddd, *J* = 3.0, 5.2, 7.7, 1H, Hγ-Azp), 4.46 (ddd, *J* = 2.7, 5.3, 7.9, 1H, Hγ-Azp), 4.36 (dd, *J* = 5.5, 8.4, 1H, Hα-Pro-NH_2), 3.94 (d, *J* = 12.1, 1H, Hδ-Azp), 3.87 – 3.78 (m, 12H, Hδ), 3.72 (d, *J* = 12.1, 1H, Hδ'), 3.66 – 3.57 (m, 10H, Hδ'), 2.54 – 2.46 (m, 2H, Hβ-Azp), 2.37 – 2.26 (m, 9H, Hβ), 2.16 – 2.08 (m, 2H, Hβ'-Azp), 2.09 (s, 3H, CH_3), 2.06 – 1.99 (m, 21H, 1 × Hβ-Pro-NH_2, Hγ), 1.94 – 1.85 (m, 10H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68091.

Dimeric azidoproline helix 12R-Azp$_2$. Yield after RP-HPLC purification: 29.0 mg (22.2 μmol, 44%). LC-MS analysis: t_R 5.45 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.77 (t, *J* = 8.5, 1H, Hα-Azp), 4.73 – 4.65 (m, 10H, Hα), 4.46 (ddd, *J* = 3.0, 5.2, 7.7, 1H, Hγ-Azp), 4.44 (ddd, *J* = 2.7, 5.3, 7.9, 1H, Hγ-Azp), 4.34 (dd, *J* = 5.5, 8.4, 1H, Hα-Pro-NH_2), 3.92 (d, *J* = 12.1, 1H, Hδ-Azp), 3.87 – 3.75 (m, 12H, Hδ), 3.70 (d, *J* = 12.1, 1H, Hδ'), 3.65 – 3.54 (m, 10H, Hδ'), 2.53 – 2.44 (m, 2H, Hβ-Azp), 2.36 – 2.24 (m, 9H, Hβ), 2.14 – 2.05 (m, 2H, Hβ'-Azp), 2.08 (s, 3H, CH_3), 2.05 – 1.95 (m, 21H, 1 × Hβ-Pro-NH_2, Hγ), 1.94 – 1.83 (m, 10H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68079.

Dimeric azidoproline helix 13R-Azp$_2$. Yield after RP-HPLC purification: 46.9 mg (35.9 μmol, 72%). LC-MS analysis: t_R 5.52 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.77 (t, *J* = 7.9, 1H, Hα-Azp), 4.73 (t, *J* = 8.0, 1H, Hα-Azp), 4.88 – 4.69 (m, 9H, Hα), 4.46 (ddd, *J* = 3.1, 5.3, 8.0, 1H, Hγ-Azp), 4.42 (ddd, *J* = 3.1, 5.4, 7.9, 1H, Hγ-Azp), 4.31 (dd, *J* = 5.3, 8.4, 1H, Hα-Pro-NH_2), 3.90 (d, *J* = 11.7, 1H, Hδ-Azp), 3.85 – 3.73 (m, 12H, Hδ), 3.68 (d, *J* = 11.7, 1H, Hδ'), 3.62 – 3.52 (m, 10H, Hδ'), 2.50 – 2.42 (m, 2H, Hβ-Azp), 2.34 – 2.21 (m, 9H, Hβ), 2.09 (ddd, *J* = 5.2, 7.9, 13.2, 1H, Hβ'-Azp), 2.06 – 2.02 (m, 1H, Hβ'-Azp), 2.05 (s, 3H, CH_3), 2.02 – 1.95 (m, 21H, 1 × Hβ-Pro-NH_2, Hγ), 1.91 – 1.81 (m, 10H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68176.

Dimeric azidoproline helix 14R-Azp$_2$. Yield after RP-HPLC purification: 33.0 mg (25.3 μmol, 51%). LC-MS analysis: t_R 5.64 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.74 – 4.65 (m, 11H, Hα), 4.51 – 4.47 (m, 2H, Hγ-Azp), 4.35 (dd, *J* = 5.5, 8.6, 1H, Hα-Pro-NH_2), 3.93 (d, *J* = 12.2, 1H, Hδ-Azp), 3.87 – 3.77 (m, 12H, Hδ), 3.67 – 3.57 (m, 11H, Hδ'), 2.48 (ddd, *J* = 2.3, 7.7, 11.0, 2H, Hβ-Azp), 2.38 – 2.25 (m, 9H, Hβ), 2.14 – 2.07 (m, 2H, Hβ'-Azp), 2.08 (s, 3H, CH_3), 2.06 – 1.96 (m, 21H, 1 × Hβ-Pro-NH_2, Hγ), 1.95 – 1.83 (m, 10H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68115.

Dimeric azidoproline helix 15R-Azp$_2$. Yield after RP-HPLC purification: 36.6 mg (28.0 μmol, 56%). LC-MS analysis: t_R 5.52 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.8 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.72 – 4.66 (m, 11H, Hα), 4.51 – 4.47 (m, 2H, Hγ-Azp), 4.34 (dd, *J* = 5.3, 8.4, 1H, Hα-Pro-NH_2), 3.93 (d, *J* = 10.5, 1H, Hδ-Azp), 3.88 – 3.77 (m, 12H, Hδ), 3.65 – 3.57 (m, 11H, Hδ'), 2.51 – 2.45 (m, 2H, Hβ-Azp), 2.37 – 2.25 (m, 9H, Hβ), 2.13 – 2.06 (m, 2H, Hβ'-Azp), 2.08 (s, 3H, CH_3), 2.06 – 1.97 (m, 21H, 1 × Hβ-Pro-NH_2, Hγ), 1.95 – 1.84 (m, 10H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68176.

Dimeric azidoproline helix 16R-Azp$_2$. Yield after RP-HPLC purification: 16.8 mg (10.5 μmol, 21%). LC-MS analysis: t_R 5.65 min (gradient 10 to 50% B). ESI-MS *m/z*: 1598.6 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.90 –

4.69 (m, 14H, Hα), 4.61 – 4.54 (m, 1H, Hγ-Azp), 4.54 – 4.48 (m, 1H, Hγ-Azp), 4.42 (dd, *J* = 5.3, 8.3, 1H, Hα-Pro-NH_2), 4.02 (d, *J* = 11.3, 1H, Hδ-Azp), 3.96 – 3.82 (m, 14H, Hδ-Azp, Hδ), 3.76 – 3.58 (m, 15H, Hδ'), 2.61-2.50 (m, 2H, Hβ-Azp), 2.48 – 2.25 (m, 13H, Hβ), 2.16 (s, 3H, CH_3), 2.23 – 2.02 (m, 29H, 1 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 2.02 – 1.83 (m, 12H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{77}H_{108}N_{22}O_{16}$ + H^+: 1597.83864, obsd 1597.83828.

Dimeric azidoproline helix 17R-Azp$_2$. Yield after RP-HPLC purification: 61.4 mg (32.5 μmol, 65%). LC-MS analysis: t_R 5.77 min (gradient 10 to 50% B). ESI-MS *m/z*: 1888.8 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.74 – 4.61 (m, 17H, Hα), 4.53 – 4.48 (m, 1H, Hγ-Azp), 4.48 – 4.41 (m, 1H, Hγ-Azp), 4.35 (dd, *J* = 5.3, 8.3, 1H, Hα-Pro-NH_2), 3.94 (d, *J* = 11.7, 1H, Hδ-Azp), 3.90 – 3.76 (m, 17H, Hδ-Azp, Hδ), 3.70 – 3.50 (m, 18H, Hδ'), 2.57 -2.44 (m, 2H, Hβ-Azp), 2.41 – 2.16 (m, 16H, Hβ), 2.08 (s, 3H, CH_3), 2.15 – 1.96 (m, 35H, 1 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 1.96 – 1.80 (m, 15H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{92}H_{129}N_{25}O_{19}$ + H^+: 1888.99693, obsd 1888.99568.

Dimeric azidoproline helix 18R-Azp$_2$. Yield after RP-HPLC purification: 58.8 mg (27.0 μmol, 54%). LC-MS analysis: t_R 5.86 min (gradient 10 to 50% B). ESI-MS *m/z*: 1091.7 $[M + 2H]^{2+}$. ^{1}H NMR (400 MHz, D_2O) δ 4.74 – 4.61 (m, 20H, Hα), 4.53 – 4.47 (m, 1H, Hγ-Azp), 4.47 – 4.41 (m, 1H, Hγ-Azp), 4.34 (dd, *J* = 5.6, 8.3, 1H, Hα-Pro-NH_2), 3.93 (d, *J* = 11.9, 1H, Hδ-Azp), 3.89 – 3.74 (m, 20H, Hδ-Azp, Hδ), 3.69 – 3.46 (m, 21H, Hδ'), 2.55 -2.41 (m, 2H, Hβ-Azp), 2.41 – 2.19 (m, 19H, Hβ), 2.08 (s, 3H, CH_3), 2.17 – 1.95 (m, 41H, 1 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 1.94 – 1.78 (m, 18H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{107}H_{150}N_{28}O_{22}$ + $2H^+$: 1090.58125, obsd 1090.58260.

Trimeric azidoproline helix 19R-Azp$_3$. Yield after RP-HPLC purification: 34.0 mg (25.2 μmol, 50%). LC-MS analysis: t_R 6.24 min (gradient 10 to 50% B). ESI-MS *m/z*: 1347.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.77 (t, *J* = 8.1, 1H, Hα-Azp), 4.73 – 4.67 (m, 10H, Hα), 4.51 – 4.47 (m, 2H, Hγ-Azp), 4.45 (ddd, *J* = 2.8, 5.3, 7.8, 1H, Hγ-Azp), 4.35 (dd, *J* = 5.5, 8.6, 1H, Hα-Pro-NH_2), 3.93 (d, *J* = 12.2, 2H, Hδ-Azp), 3.87 – 3.78 (m, 12H, Hδ), 3.72 (d, *J* = 11.9, 1H, Hδ'-Azp), 3.66 – 3.57 (m, 9H, Hδ'), 2.54 – 2.46 (m, 3H, Hβ-Azp), 2.37 – 2.25 (m, 8H, Hβ), 2.16 – 2.07 (m, 3H, Hβ'-Azp), 2.09 (s, 3H, CH_3), 2.06 – 1.98 (m, 19H, 1 × Hβ-Pro-NH_2, Hγ), 1.95 – 1.84 (m, 9H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{86}N_{22}O_{13}$ + H^+: 1347.68175, obsd 1347.68274.

Tetrameric azidoproline helix 20R-Azp$_4$. Yield after RP-HPLC purification: 14.3 mg (10.3 μmol, 21%). LC-MS analysis: t_R 6.72 min (gradient 10 to 50% B). ESI-MS *m/z*: 1388.8 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.88 – 4.70 (m, 11H, Hα), 4.57 – 4.51 (m, 3H, Hγ-Azp), 4.51 – 4.46 (m, 1H, Hγ-Azp), 4.40 (dd, *J* = 5.5, 8.4, 1H, Hα-Pro-NH_2), 3.98 (d, *J* = 11.4, 3H, Hδ-Azp), 3.94 – 3.79 (m, 9H, Hδ-Azp, Hδ), 3.72 – 3.59 (m, 12H, Hδ'), 2.61 -2.47 (m, 4H, Hβ-Azp), 2.46 – 2.26 (m, 8H, Hβ), 2.14 (s, 3H, CH_3), 2.23 – 2.02 (m, 21H, 4 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 2.01 – 1.84 (m, 7H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{85}N_{25}O_{13}$ + H^+: 1388.68314, obsd 1388.68314.

Monomeric azidoproline helix 1S-Azp. Yield after RP-HPLC purification: 50.0 mg (39.5 μmol, 79%). LC-MS analysis: t_R 4.74 min (gradient 10 to 50% B). ESI-MS *m/z*: 1265.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.82 – 4.67 (m, 11H, Hα), 4.46 (dt, *J* = 5.2, 10.6, 1H, Hγ-Azp), 4.38 (dd, *J* = 5.6, 8.7, 1H, Hα-Pro-NH_2), 3.97 (dd, *J* = 6.3, 11.2, 1H, Hδ-Azp), 3.89 – 3.77 (m, 11H, Hδ), 3.70 – 3.53 (m, 12H, Hδ', Hδ'-Azp), 2.73 (ddd, *J* = 6.2, 9.2, 13.2, 1H, Hβ-Azp), 2.41 – 2.24 (m, 11H, Hβ), 2.10 (s, 3H, CH_3), 2.09 – 1.98 (m, 24H, 1 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 1.98 – 1.84 (m, 10H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{68}N_{16}O_{13}$ + H^+: 1265.67895, obsd 1265.67881.

Monomeric azidoproline helix 2S-Azp. Yield after RP-HPLC purification: 22.9 mg (18.0 μmol, 36%). LC-MS analysis: t_R 4.83 min (gradient 10 to 50% B). ESI-MS *m/z*: 1265.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.82 – 4.68 (m, 11H, Hα), 4.46 (dt, *J* = 5.9, 12.0, 1H, Hγ-Azp), 4.40 (dd, *J* = 5.4, 8.5, 1H, Hα-Pro-NH_2), 4.19 (dd, *J* = 6.6, 10.9, 1H, Hδ-Azp), 3.92 – 3.79 (m, 11H, Hδ), 3.73 – 3.60 (m, 11H, Hδ'), 3.56 (dd, *J* = 5.7, 10.7, 1H, Hδ'-Azp), 2.76 (ddd, *J* = 6.8, 8.6, 14.0, 1H, Hβ-Azp), 2.44 – 2.27 (m, 11H, Hβ), 2.12 (s, 3H, CH_3), 2.11 – 2.00 (m, 24H, 1 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 1.99 – 1.87 (m, 10H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{68}N_{16}O_{13}$ + H^+: 1265.67895, obsd 1265.67888.

Monomeric azidoproline helix 3S-Azp. Yield after RP-HPLC purification: 23.6 mg (18.6 μmol, 37%). LC-MS analysis: t_R 4.79 min (gradient 10 to 50% B). ESI-MS *m/z*: 1265.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.76 – 4.66 (m, 11H, Hα), 4.46 (dt, *J* = 6.0, 11.9, 1H, Hγ-Azp), 4.39 (dd, *J* = 5.4, 8.5, 1H, Hα-Pro-NH_2), 4.19 (dd, *J* = 6.4, 11.0, 1H, Hδ-Azp), 3.90 – 3.79 (m, 11H, Hδ), 3.72 – 3.59 (m, 11H, Hδ'), 3.57 (dd, *J* = 5.7, 10.9, 1H, Hδ'-Azp), 2.75 (ddd, *J* = 6.3, 8.7, 14.0, 1H, Hβ-Azp), 2.42 – 2.27 (m, 11H, Hβ), 2.12 (s, 3H, CH_3), 2.10 – 1.99 (m, 24H, 1 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 1.99 – 1.84 (m, 10H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{68}N_{16}O_{13}$ + H^+: 1265.67895, obsd 1265.67897.

Monomeric azidoproline helix 4S-Azp. Yield after RP-HPLC purification: 22.3 mg (17.6 μmol, 35%). LC-MS analysis: t_R 4.80 min (gradient 10 to 50% B). ESI-MS *m/z*: 1265.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.93 – 4.67 (m, 11H, Hα), 4.46 (dt, *J* = 6.5, 12.6, 1H, Hγ-Azp), 4.30 (dd, *J* = 5.3, 8.5, 1H, Hα-Pro-NH_2), 4.22 (dd, *J* = 6.5, 10.9, 1H, Hδ-Azp), 3.93 – 3.81 (m, 11H, Hδ), 3.74 – 3.60 (m, 11H, Hδ'), 3.56 (dd, *J* = 5.5, 10.6, 1H, Hδ'-Azp), 2.78 (ddd, *J* = 6.6, 8.9, 13.5, 1H, Hβ-Azp), 2.46 – 2.26 (m, 11H, Hβ), 2.13 (s, 3H, CH_3), 2.11 – 1.01 (m, 24H, 1 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 2.01 – 1.84 (m, 10H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{68}N_{16}O_{13}$ + H^+: 1265.67895, obsd 1265.67961.

Dimeric azidoproline helix 5S-Azp$_2$. Yield after RP-HPLC purification: 31.8 mg (24.1 μmol, 48%). LC-MS analysis: t_R 5.23 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.76 – 4.68 (m, 11H, Hα), 4.45 – 4.37 (m, 2H, Hγ-Azp), 4.36 (dd, *J* = 5.4, 8.6, 1H, Hα-Pro-NH_2), 4.13 (dd, *J* = 6.6, 10.9, 1H, Hδ-Azp), 3.95 (dd, *J* = 6.1, 11.2, 1H, Hδ-Azp), 3.87 – 3.77 (m, 10H, Hδ), 3.68 – 3.53 (m, 11H, Hδ', Hδ'-Azp), 3.49 (dd, *J* = 6.1, 10.9, 1H, Hδ'-Azp), 2.77 – 2.67 (m, 2H, Hβ-Azp), 2.37 – 2.27 (m, 10H, Hβ), 2.09 (s, 3H, CH_3), 2.08 – 1.98 (m, 23H, 2 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 1.97 – 1.85 (m, 9H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68140.

Dimeric azidoproline helix 6S-Azp$_2$. Yield after RP-HPLC purification: 37.5 mg (28.7 μmol, 57%). LC-MS analysis: t_R 5.06 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.76 (dd, *J* = 3.5, 9.0, 2H, Hα-Azp), 4.74 – 4.67 (m, 9H, Hα), 4.46 – 4.38 (m, 2H, Hγ-Azp), 4.36 (dd, *J* = 5.4, 8.6, 1H, Hα-Pro-NH_2), 4.18 (dd, *J* = 6.6, 11.0, 1H, Hδ-Azp), 3.94 (dd, *J* = 6.2, 11.3, 1H, Hδ-Azp), 3.87 – 3.74 (m, 10H, Hδ), 3.68 – 3.50 (m, 12H, Hδ', Hδ'-Azp), 2.76 – 2.67 (m, 2H, Hβ-Azp), 2.38 – 2.26 (m, 10H, Hβ), 2.09 (s, 3H, CH_3), 2.07 – 1.97 (m, 23H, 2 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 1.97 – 1.84 (m, 9H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68188.

Dimeric azidoproline helix 7S-Azp$_2$. Yield after RP-HPLC purification: 19.4 mg (14.8 μmol, 30%). LC-MS analysis: t_R 5.14 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.77 – 4.66 (m, 11H, Hα), 4.74 – 4.67 (m, 9H, Hα), 4.46 – 4.38 (m, 2H, Hγ-Azp), 4.39 (dd, *J* = 5.4, 8.6, 1H, Hα-Pro-NH_2), 4.19 (dd, *J* = 6.4, 11.0, 1H, Hδ-Azp), 3.98 (dd, *J* = 6.3, 11.3, 1H, Hδ-Azp), 3.90 – 3.77 (m, 10H, Hδ), 3.71 – 3.53 (m, 12H, Hδ', Hδ'-Azp), 2.79 – 2.70 (m, 2H, Hβ-Azp), 2.41 – 2.28 (m, 10H, Hβ), 2.10 (s, 3H, CH_3), 2.10 –

2.00 (m, 23H, 2 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 2.00 – 1.86 (m, 9H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68140.

Dimeric azidoproline helix 8S-Azp$_2$. Yield after RP-HPLC purification: 4.9 mg (3.8 μmol, 8%). LC-MS analysis: t_R 5.21 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.77 – 4.66 (m, 11H, Hα), 4.44 (tt, *J* = 6.1, 12.0, 2H, Hγ-Azp), 4.39 (dd, *J* = 5.4, 8.6, 1H, Hα-Pro-NH_2), 4.19 (dd, *J* = 6.4, 11.0, 1H, Hδ-Azp), 3.98 (dd, *J* = 6.3, 11.3, 1H, Hδ-Azp), 3.90 – 3.77 (m, 10H, Hδ), 3.71 – 3.53 (m, 12H, Hδ', Hδ'-Azp), 2.80 – 2.70 (m, 2H, Hβ-Azp), 2.41 – 2.28 (m, 10H, Hβ), 2.12 (s, 3H, CH_3), 2.10 – 2.00 (m, 23H, 2 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 2.00 – 1.86 (m, 9H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68164.

Dimeric azidoproline helix 9S-Azp$_2$. Yield after RP-HPLC purification: 20.2 mg (15.5 μmol, 31%). LC-MS analysis: t_R 5.12 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.78 – 4.66 (m, 11H, Hα), 4.43 (ddd, *J* = 6.1, 12.0, 18.1, 2H, Hγ-Azp), 4.38 (dd, *J* = 5.4, 8.6, 1H, Hα-Pro-NH_2), 4.17 (dd, *J* = 6.5, 11.0, 1H, Hδ-Azp), 3.97 (dd, *J* = 6.3, 11.2, 1H, Hδ-Azp), 3.89 – 3.76 (m, 10H, Hδ), 3.69 – 3.48 (m, 12H, Hδ', Hδ'-Azp), 2.78 – 2.69 (m, 2H, Hβ-Azp), 2.41 – 2.27 (m, 10H, Hβ), 2.10 (s, 3H, CH_3), 2.09 – 1.99 (m, 23H, 2 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ),1.98 – 1.84 (m, 9H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68152.

Dimeric azidoproline helix 10S-Azp$_2$. Yield after RP-HPLC purification: 26.2 mg (20.1 μmol, 40%). LC-MS analysis: t_R 5.12 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.79 – 4.64 (m, 11H, Hα), 4.42 (ddd, *J* = 6.3, 12.1, 18.1, 2H, Hγ-Azp), 4.37 (dd, *J* = 5.4, 8.6, 1H, Hα-Pro-NH_2), 4.16 (dd, *J* = 6.6, 11.3, 1H, Hδ-Azp), 3.96 (dd, *J* = 6.3, 11.2, 1H, Hδ-Azp), 3.87 – 3.75 (m, 10H, Hδ), 3.68 – 3.50 (m, 12H, Hδ', Hδ'-Azp), 2.77 – 2.68 (m, 2H, Hβ-Azp), 2.42 – 2.26 (m, 10H, Hβ), 2.10 (s, 3H, CH_3), 2.08 – 1.99 (m, 23H, 2 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 1.98 – 1.83 (m, 9H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68127.

Dimeric azidoproline helix 11S-Azp$_2$. Yield after RP-HPLC purification: 26.4 mg (20.2 μmol, 40%). LC-MS analysis: t_R 5.18 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.79 – 4.65 (m, 11H, Hα), 4.43 (tt, *J* = 5.4, 8.6, 2H, Hγ-Azp), 4.37 (dd, *J* = 5.4, 8.6, 1H, Hα-Pro-NH_2), 4.17 (dd, *J* = 6.7, 11.4, 1H, Hδ-Azp), 3.96 (dd, *J* = 6.4, 11.2, 1H, Hδ-Azp), 3.89 – 3.75 (m, 10H, Hδ), 3.70 – 3.44 (m, 12H, Hδ', Hδ'-Azp), 2.78 – 2.68 (m, 2H, Hβ-Azp), 2.45 – 2.24 (m, 10H, Hβ), 2.10 (s, 3H, CH_3), 2.09 – 1.98 (m, 23H, 2 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 1.98 – 1.84 (m, 9H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68188.

Dimeric azidoproline helix 12S-Azp$_2$. Yield after RP-HPLC purification: 17.8 mg (13.6 μmol, 27%). LC-MS analysis: t_R 5.14 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.80 – 4.67 (m, 11H, Hα), 4.44 (ddd, *J* = 6.1, 12.2, 18.0, 2H, Hγ-Azp), 4.38 (dd, *J* = 5.4, 8.5, 1H, Hα-Pro-NH_2), 4.18 (dd, *J* = 6.6, 11.1, 1H, Hδ-Azp), 3.98 (dd, *J* = 6.3, 11.3, 1H, Hδ-Azp), 3.90 – 3.76 (m, 10H, Hδ), 3.70 – 3.52 (m, 12H, Hδ', Hδ'-Azp), 2.74 (tdd, *J* = 4.0, 6.3, 9.3, 2H, Hβ-Azp), 2.39 – 2.27 (m, 10H, Hβ), 2.11 (s, 3H, CH_3), 2.10 – 2.00 (m, 23H, 2 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 1.99 – 1.86 (m, 9H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68140.

Dimeric azidoproline helix 13S-Azp$_2$. Yield after RP-HPLC purification: 42.3 mg (32.4 μmol, 65%). LC-MS analysis: t_R 5.05 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.75 –

4.67 (m, 11H, Hα), 4.44 (dt, *J* = 6.3, 12.8, 2H, Hγ-Azp), 4.39 (dd, *J* = 5.2, 8.6, 1H, Hα-Pro-NH_2), 4.19 (dd, *J* = 6.6, 11.0, 1H, Hδ-Azp), 3.97 (dd, *J* = 6.3, 11.3, 1H, Hδ-Azp), 3.89 – 3.75 (m, 10H, Hδ), 3.71 – 3.46 (m, 12H, Hδ', Hδ'-Azp), 2.74 (ddt, *J* = 6.6, 8.9, 13.6, 2H, Hβ-Azp), 2.42 – 2.25 (m, 10H, Hβ), 2.11 (s, 3H, CH_3), 2.09 – 1.99 (m, 23H, 2 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 1.99 – 1.85 (m, 9H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68127.

Dimeric azidoproline helix 14S-Azp$_2$. Yield after RP-HPLC purification: 21.2 mg (16.2 μmol, 32%). LC-MS analysis: t_R 5.21 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.80 – 4.65 (m, 11H, Hα), 4.44 (ddd, *J* = 6.0, 11.8, 18.2, 2H, Hγ-Azp), 4.38 (dd, *J* = 5.4, 8.5, 1H, Hα-Pro-NH_2), 4.17 (dt, *J* = 6.7, 11.4, 2H, Hδ-Azp), 3.89 – 3.76 (m, 10H, Hδ), 3.70 – 3.58 (m, 10H, Hδ'), 3.54 (ddd, *J* = 3.1, 5.0, 10.5, 2H, Hδ'-Azp), 2.78 – 2.69 (m, 2H, Hβ-Azp), 2.41 – 2.27 (m, 10H, Hβ), 2.10 (s, 3H, CH_3), 2.09 – 1.98 (m, 23H, 2 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 1.98 – 1.85 (m, 9H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68127.

Dimeric azidoproline helix 15S-Azp$_2$. Yield after RP-HPLC purification: 40.6 mg (31.1 μmol, 62%). LC-MS analysis: t_R 5.14 min (gradient 10 to 50% B). ESI-MS *m/z*: 1306.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.78 – 4.62 (m, 11H, Hα), 4.43 (ddd, *J* = 5.8, 11.4, 17.1, 2H, Hγ-Azp), 4.37 (dd, *J* = 5.3, 8.7, 1H, Hα-Pro-NH_2), 4.16 (ddd, *J* = 6.5, 11.1, 14.1, 2H, Hδ-Azp), 3.86 – 3.75 (m, 10H, Hδ), 3.68 – 3.48 (m, 12H, Hδ', Hδ'-Azp), 2.72 (ddd, *J* = 6.2, 9.0, 13.4, 2H, Hβ-Azp), 2.36 – 2.25 (m, 10H, Hβ), 2.08 (s, 3H, CH_3), 2.07 – 1.97 (m, 23H, 2 × Hβ'-Azp, 1 × HβPro-NH_2, Hγ), 1.97 – 1.84 (m, 9H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{87}N_{19}O_{13}$ + H^+: 1306.68035, obsd 1306.68176.

Dimeric azidoproline helix 16S-Azp$_2$. Yield after RP-HPLC purification: 35.3 mg (22.1 μmol, 44%). LC-MS analysis: t_R 5.32 min (gradient 10 to 50% B). ESI-MS *m/z*: 1597.6 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.82 – 4.67 (m, 14H, Hα), 4.44 (dt, *J* = 6.6, 12.9, 2H, Hγ-Azp), 4.40 (dd, *J* = 5.3, 8.9, 1H, Hα-Pro-NH_2), 4.19 (dd, *J* = 6.6, 11.0, 1H, Hδ-Azp), 3.97 (dd, *J* = 6.3, 11.3, 1H, Hδ-Azp), 3.89 – 3.78 (m, 13H, Hδ), 3.69 – 3.57 (m, 14H, Hδ'), 3.54 (dd, *J* = 5.7, 10.5, 1H, Hδ'-Azp), 2.74 (ddt, *J* = 6.7, 8.8, 13.6, 2H, Hβ-Azp), 2.40 – 2.25 (m, 12H, Hβ), 2.11 (s, 3H, CH_3), 2.10 – 1.99 (m, 29H, 2 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 1.98 – 1.85 (m, 13H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{77}H_{108}N_{22}O_{16}$ + H^+: 1597.83864, obsd 1597.83844.

Dimeric azidoproline helix 17S-Azp$_2$. Yield after RP-HPLC purification: 57.9 mg (30.7 μmol, 61%). LC-MS analysis: t_R 5.44 min (gradient 10 to 50% B). ESI-MS *m/z*: 1888.7 $[M + H]^+$.^{1}H NMR (400 MHz, D_2O) δ 4.83 – 4.62 (m, 17H, Hα), 4.38 (dt, *J* = 6.7, 12.8, 2H, Hγ-Azp), 4.35 (dd, *J* = 5.3, 8.8, 1H, Hα-Pro-NH_2), 4.15 (dd, *J* = 6.4, 10.5, 1H, Hδ-Azp), 3.92 (dd, *J* = 6.2, 11.2, 1H, Hδ-Azp), 3.87 – 3.74 (m, 16H, Hδ), 3.67 – 3.55 (m, 17H, Hδ'), 3.49 (dd, *J* = 5.6, 10.3, 1H, Hδ'-Azp), 2.69 (ddt, *J* = 6.0, 7.5, 13.5, 2H, Hβ-Azp), 2.43 – 2.20 (m, 15H, Hβ), 2.06 (s, 3H, CH_3), 2.05 – 1.94 (m, 35H, 2 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 1.94 – 1.81 (m, 16H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{92}H_{129}N_{25}O_{19}$ + H^+: 1888.99693, obsd 1888.99924.

Dimeric azidoproline helix 18S-Azp$_2$. Yield after RP-HPLC purification: 42.8 mg (19.6 μmol, 39%). LC-MS analysis: t_R 5.54 min (gradient 10 to 50% B). ESI-MS *m/z*: 1090.4 $[M + 2H]^{2+}$.^{1}H NMR (400 MHz, D_2O) δ 4.80 – 4.60 (m, 20H, Hα), 4.40 (dt, *J* = 6.5, 12.8, 2H, Hγ-Azp), 4.36 (dd, *J* = 5.3, 8.9, 1H, Hα-Pro-NH_2), 4.16 (dd, *J* = 6.6, 11.0, 1H, Hδ-Azp), 3.94 (dd, *J* = 6.3, 11.2, 1H, Hδ-Azp), 3.86 – 3.75 (m, 19H, Hδ), 3.68 – 3.54 (m, 20H, Hδ'), 3.51 (dd, *J* = 5.6, 10.4, 1H, Hδ'-Azp), 2.71 (ddt, *J* = 5.6, 7.6, 13.2, 2H, Hβ-Azp), 2.43 – 2.23 (m, 18H, Hβ), 2.08 (s, 3H, CH_3), 2.06 – 1.95 (m, 41H, 2 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 1.95 – 1.80 (m, 19H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{107}H_{150}N_{28}O_{22}$ + 2H^+: 1090.58125, obsd 1090.58221.

Trimeric azidoproline helix 19S-Azp$_3$. Yield after RP-HPLC purification: 17.5 mg (13.0 µmol, 26%). LC-MS analysis: t_R 5.57 min (gradient 10 to 50% B). ESI-MS *m/z*: 1347.7 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.78 – 4.66 (m, 11H, Hα), 4.47 – 4.41 (m, 3H, Hγ-Azp), 4.38 (dd, *J* = 5.4, 8.5, 1H, Hα-Pro-NH_2), 4.17 (ddd, *J* = 3.1, 5.6, 11.3, 2H, Hδ-Azp), 3.97 (dd, *J* = 6.2, 11.3, 1H, Hδ-Azp), 3.90 – 3.76 (m, 9H, Hδ), 3.70 – 3.51 (m, 12H, Hδ', Hδ'-Azp), 2.78 – 2.69 (m, 3H, Hβ-Azp), 2.39 – 2.27 (m, 9H, Hβ), 2.11 (s, 3H, CH_3), 2.10 – 1.99 (m, 21H, 3 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 1.99 – 1.86 (m, 8H, 1 × Hβ'-Pro-NH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{86}N_{22}O_{13}$ + H^+: 1347.68175, obsd 1347.68274

Tetrameric azidoproline helix 20S-Azp$_4$. Yield after RP-HPLC purification: 13.7 mg (9.9 µmol, 20%). LC-MS analysis: t_R 5.98 min (gradient 10 to 50% B). ESI-MS *m/z*: 1388.8 $[M + H]^+$. ^{1}H NMR (400 MHz, D_2O) δ 4.84 – 4.63 (m, 11H, Hα), 4.50 – 4.36 (m, 5H, Hγ-Azp, Hα-Pro-NH_2), 4.25 – 4.13 (m, 3H, Hδ-Azp), 3.97 (dd, *J* = 6.3, 11.2, 1H, Hδ-Azp), 3.92 – 3.76 (m, 8H, Hδ), 3.72 – 3.46 (m, 12H, Hδ'), 2.81 -2.67 (m, 4H, Hβ-Azp), 2.43 – 2.25 (m, 8H, Hβ), 2.12 (s, 3H, CH_3), 2.10 – 2.00 (m, 21H, 4 × Hβ'-Azp, 1 × Hβ-Pro-NH_2, Hγ), 2.00 – 1.87 (m, 7H, 1 × Hβ'-ProNH_2, Hβ). HRMS *m/z* calcd for $C_{62}H_{85}N_{25}O_{13}$ + H^+: 1388.68314, obsd 1388.68298.

General procedure for the functionalization of azidoproline peptides with LHA.
To a solution of the desired azidoproline peptide (5.0 µmol) and **LHA** (1.2 eq. per azide, 6 µmol, 2.9 mg) in a mixture of degassed *t*BuOH/MeCN/H_2O (2/2/1; v/v/v, 500 µL) were added sodium ascorbate (2.5 eq. per azide, 50 µL of a 0.25M solution in H_2O) and $CuSO_4$ (0.5 eq. per azide, 25 µL of a 0.1M solution in H_2O). The reaction mixture was stirred and heated at 60 °C for 3 h. The mixture was evaporated, redissolved H_2O/CH_3CN (1/1; v/v, 1 mL) and filtrated. The crude products were analyzed by LC-MS and purified by semi-preparative RP-HPLC (linear gradient of 5.0 CV; 40 to 80% B). Evaporation and lyophilization of the combined fractions from Dioxane/H_2O (1/1; v/v) furnished the ligands as yellow amorphous powders.

Monomeric ligand 1R-LHA. Yield after RP-HPLC purification: 2.0 mg (1.1 µmol, 21%). LC-MS analysis: t_R 6.58 min (gradient 10 to 90% B). ESI-MS *m/z*: 1747.6 $[M + H]^+$. HRMS *m/z* calcd for $C_{85}H_{114}N_{22}O_{15}S_2$ + H^+: 1747.83482, obsd 1747.83601.

Monomeric ligand 2R-LHA. Yield after RP-HPLC purification: 1.6 mg (0.9 µmol, 17%). LC-MS analysis: t_R 6.54 min (gradient 10 to 90% B). ESI-MS *m/z*: 1747.6 $[M + H]^+$. HRMS *m/z* calcd for $C_{85}H_{114}N_{22}O_{15}S_2$ + H^+: 1747.83482, obsd 1747.83581.

Monomeric ligand 3R-LHA. Yield after RP-HPLC purification: 1.1 mg (0.6 µmol, 12%). LC-MS analysis: t_R 6.40 min (gradient 10 to 90% B). ESI-MS *m/z*: 1747.6 $[M + H]^+$. HRMS *m/z* calcd for $C_{85}H_{114}N_{22}O_{15}S_2$ + H^+: 1747.83482, obsd 1747.83570.

Monomeric ligand 4R-LHA. Yield after RP-HPLC purification: 4.1 mg (2.2 µmol, 44%). LC-MS analysis: t_R 6.50 min (gradient 10 to 90% B). ESI-MS *m/z*: 1747.7 $[M + H]^+$. HRMS *m/z* calcd for $C_{85}H_{114}N_{22}O_{15}S_2$ + H^+: 1747.83482, obsd 1747.83561.

Dimeric ligand 5R-LHA$_2$. Yield after RP-HPLC purification: 2.2 mg (1.0 µmol, 19%). LC-MS analysis: t_R 8.24 min (gradient 10 to 90% B). ESI-MS *m/z*: 1136.4 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4$ + $2H^+$: 1135.99968, obsd 1136.00061.

Dimeric ligand 6R-LHA$_2$. Yield after RP-HPLC purification: 2.7 mg (1.2 μmol, 24%). LC-MS analysis: t_R 8.20 min (gradient 10 to 90% B). ESI-MS *m/z*: 1136.5 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1136.00098.

Dimeric ligand 7R-LHA$_2$. Yield after RP-HPLC purification: 2.1 mg (0.9 μmol, 19%). LC-MS analysis: t_R 8.17 min (gradient 10 to 90% B). ESI-MS *m/z*: 1136.5 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1136.00012.

Dimeric ligand 8R-LHA$_2$. Yield after RP-HPLC purification: 2.4 mg (1.1 μmol, 21%). LC-MS analysis: t_R 8.06 min (gradient 10 to 90% B). ESI-MS *m/z*: 1136.1 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1136.00024.

Dimeric ligand 9R-LHA$_2$. Yield after RP-HPLC purification: 2.3 mg (1.0 μmol, 20%). LC-MS analysis: t_R 7.98 min (gradient 10 to 90% B). ESI-MS *m/z*: 1136.1 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1135.99927.

Dimeric ligand 10R-LHA$_2$. Yield after RP-HPLC purification: 2.5 mg (1.1 μmol, 22%). LC-MS analysis: t_R 7.94 min (gradient 10 to 90% B). ESI-MS *m/z*: 1136.4 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1136.00110.

Dimeric ligand 11R-LHA$_2$. Yield after RP-HPLC purification: 2.3 mg (1.0 μmol, 20%). LC-MS analysis: t_R 7.98 min (gradient 10 to 90% B). ESI-MS *m/z*: 1136.2 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1136.00037.

Dimeric ligand 12R-LHA$_2$. Yield after RP-HPLC purification: 1.8 mg (0.8 μmol, 16%). LC-MS analysis: t_R 7.89 min (gradient 10 to 90% B). ESI-MS *m/z*: 1135.9 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1136.00171.

Dimeric ligand 13R-LHA$_2$. Yield after RP-HPLC purification: 2.8 mg (1.2 μmol, 25%). LC-MS analysis: t_R 7.94 min (gradient 10 to 90% B). ESI-MS *m/z*: 1135.7 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1136.00159.

Dimeric ligand 14R-LHA$_2$. Yield after RP-HPLC purification: 2.2 mg (1.0 μmol, 19%). LC-MS analysis: t_R 8.11 min (gradient 10 to 90% B ESI-MS *m/z*: 1136.5 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1136.00110.

Dimeric ligand 15R-LHA$_2$. Yield after RP-HPLC purification: 3.0 mg (1.3 μmol, 26%). LC-MS analysis: t_R 8.03 min (gradient 10 to 90% B ESI-MS *m/z*: 1136.7 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1136.00110.

Dimeric ligand 16R-LHA$_2$. Yield after RP-HPLC purification: 1.9 mg (0.7 μmol, 14%). LC-MS analysis: t_R 7.76 min (gradient 10 to 90% B). ESI-MS *m/z*: 1281.5 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{123}H_{160}N_{34}O_{20}S_4 + 2H^+$: 1281.57882, obsd 1281.57994.

Dimeric ligand 17R-LHA$_2$. Yield after RP-HPLC purification: 3.6 mg (1.2 μmol, 24%). LC-MS analysis: t_R 7.56 min (gradient 10 to 90% B). ESI-MS *m/z*: 1427.1 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{138}H_{181}N_{37}O_{23}S_4 + 2H^+$: 1427.15797, obsd 1427.15894.

Dimeric ligand 18R-LHA$_2$. Yield after RP-HPLC purification: 2.8 mg (0.8 μmol, 17%). LC-MS analysis: t_R 7.43 min (gradient 10 to 90% B). ESI-MS *m/z*: 1572.7 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{153}H_{202}N_{40}O_{26}S_4 + 2H^+$: 1572.73712, obsd 1572.73759.

Trimeric ligand 19R-LHA$_3$. Yield after RP-HPLC purification: 2.7 mg (1.0 μmol, 19%). LC-MS analysis: t_R 9.51 min (gradient 10 to 90% B). ESI-MS *m/z*: 1397.9 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{131}H_{164}N_{40}O_{19}S_6 + 2H^+$: 1397.57831, obsd 1397.57825.

Tetrameric ligand 20R-LHA$_4$. Yield after RP-HPLC purification: 1.4 mg (0.4 μmol, 7%). LC-MS analysis: t_R 10.91 min (gradient 10 to 90% B). ESI-MS *m/z*: 1659.6 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{154}H_{189}N_{49}O_{21}S_8 + 2H^+$: 1659.15694, obsd 1659.15793.

Monomeric ligand 1S-LHA. Yield after RP-HPLC purification: 2.2 mg (1.1 μmol, 24%). LC-MS analysis: t_R 6.48 min (gradient 10 to 90% B). ESI-MS *m/z*: 1747.7 $[M + H]^{2+}$. HRMS *m/z* calcd for $C_{85}H_{114}N_{22}O_{15}S_2 + H^+$: 1747.83482, obsd 1747.83534.

Monomeric ligand 2S-LHA. Yield after RP-HPLC purification: 1.9 mg (1.0 μmol, 20%). LC-MS analysis: t_R 6.39 min (gradient 10 to 90% B). ESI-MS *m/z*: 1747.7 $[M + H]^{2+}$. HRMS *m/z* calcd for $C_{85}H_{114}N_{22}O_{15}S_2 + H^+$: 1747.83482, obsd 1747.83455.

Monomeric ligand 3S-LHA. Yield after RP-HPLC purification: 1.9 mg (1.0 μmol, 20%). LC-MS analysis: t_R 6.32 min (gradient 10 to 90% B). ESI-MS *m/z*: 1747.5 $[M + H]^{2+}$. HRMS *m/z* calcd for $C_{85}H_{114}N_{22}O_{15}S_2 + H^+$: 1747.83482, obsd 1747.83542.

Monomeric ligand 4S-LHA. Yield after RP-HPLC purification: 1.5 mg (0.8 μmol, 16%). LC-MS analysis: t_R 6.39 min (gradient 10 to 90% B). ESI-MS *m/z*: 1747.6 $[M + H]^{2+}$. HRMS *m/z* calcd for $C_{85}H_{114}N_{22}O_{15}S_2 + H^+$: 1747.83482, obsd 1747.83532.

Dimeric ligand 5S-LHA$_2$. Yield after RP-HPLC purification: 2.9 mg (1.3 μmol, 26%). LC-MS analysis: t_R 8.01 min (gradient 10 to 90% B). ESI-MS *m/z*: 1136.3 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1135.99915.

Dimeric ligand 6S-LHA$_2$. Yield after RP-HPLC purification: 4.7 mg (2.1 μmol, 41%). LC-MS analysis: t_R 7.97 min (gradient 10 to 90% B). ESI-MS *m/z*: 1136.5 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1136.00085.

Dimeric ligand 7S-LHA$_2$. Yield after RP-HPLC purification: 3.5 mg (1.5 μmol, 31%). LC-MS analysis: t_R 8.08 min (gradient 10 to 90% B). ESI-MS *m/z*: 1136.5 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1136.00012.

Dimeric ligand 8S-LHA$_2$. Yield after RP-HPLC purification: 1.0 mg (0.4 μmol, 9%). LC-MS analysis: t_R 7.93 min (gradient 10 to 90% B). ESI-MS *m/z*: 1136.0 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1136.00085.

Dimeric ligand 9S-LHA$_2$. Yield after RP-HPLC purification: 4.4 mg (1.9 μmol, 39%). LC-MS analysis: t_R 7.86 min (gradient 10 to 90% B). ESI-MS *m/z*: 1136.4 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1136.00064.

Dimeric ligand 10S-LHA$_2$. Yield after RP-HPLC purification: 4.1 mg (1.8 μmol, 36%). LC-MS analysis: t_R 7.92 min (gradient 10 to 90% B). ESI-MS *m/z*: 1136.3 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1135.99890.

Dimeric ligand 11S-LHA$_2$. Yield after RP-HPLC purification: 3.4 mg (1.5 μmol, 30%). LC-MS analysis: t_R 7.95 min (gradient 10 to 90% B). ESI-MS *m/z*: 1136.1 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1136.00049.

Dimeric ligand 12S-LHA$_2$. Yield after RP-HPLC purification: 3.5 mg (1.5 μmol, 31%). LC-MS analysis: t_R 7.91 min (gradient 10 to 90% B ESI-MS *m/z*: 1136.3 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1135.99976.

Dimeric ligand 13S-LHA$_2$. Yield after RP-HPLC purification: 3.5 mg (1.5 μmol, 31%). LC-MS analysis: t_R 8.00 min (gradient 10 to 90% B ESI-MS *m/z*: 1137.3 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1136.00000.

Dimeric ligand 14S-LHA$_2$. Yield after RP-HPLC purification: 3.7 mg (1.6 μmol, 33%). LC-MS analysis: t_R 7.97 min (gradient 10 to 90% B). ESI-MS *m/z*: 1136.2 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1136.00073.

Dimeric ligand 15S-LHA$_2$. Yield after RP-HPLC purification: 3.2 mg (1.4 μmol, 28%). LC-MS analysis: t_R 8.07 min (gradient 10 to 90% B). ESI-MS *m/z*: 1136.5 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{108}H_{139}N_{31}O_{17}S_4 + 2H^+$: 1135.99968, obsd 1136.00000.

Dimeric ligand 16S-LHA$_2$. Yield after RP-HPLC purification: 2.9 mg (0.7 μmol, 20%). LC-MS analysis: t_R 7.77 min (gradient 10 to 90% B). ESI-MS *m/z*: 1281.5 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{123}H_{160}N_{34}O_{20}S_4 + 2H^+$: 1281.57882, obsd 1281.57958.

Dimeric ligand 17S-LHA$_2$. Yield after RP-HPLC purification: 1.3 mg (0.4 μmol, 9%). LC-MS analysis: t_R 7.61 min (gradient 10 to 90% B). ESI-MS *m/z*: 1426.9 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{138}H_{181}N_{37}O_{23}S_4 + 2H^+$: 1427.15797, obsd 1427.15883.

Dimeric ligand 18S-LHA$_2$. Yield after RP-HPLC purification: 2.9 mg (0.9 μmol, 17%). LC-MS analysis: t_R 7.46 min (gradient 10 to 90% B). ESI-MS *m/z*: 1572.5 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{153}H_{202}N_{40}O_{26}S_4 + 2H^+$: 1572.73712, obsd 1572.73805.

Trimeric ligand 19S-LHA$_3$. Yield after RP-HPLC purification: 2.7 mg (1.0 μmol, 19%). LC-MS analysis: t_R 9.50 min (gradient 10 to 90% B). ESI-MS *m/z*: 1397.9 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{131}H_{164}N_{40}O_{19}S_6 + 2H^+$: 1397.57831, obsd 1397.57898.

Tetrameric ligand 20S-LHA$_4$. Yield after RP-HPLC purification: 1.5 mg (0.4 μmol, 8%). LC-MS analysis: t_R 10.9 min (gradient 10 to 90% B). ESI-MS *m/z*: 1659.6 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{154}H_{189}N_{49}O_{21}S_8 + 2H^+$: 1659.15694, obsd 1659.15774.

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Chapter 8

Synthesis and pharmacological evaluation of dimeric FSHR antagonists

Introduction

The gonadotropin receptors,[1,2] including the luteinizing hormone/choriogonadotropin receptor (LH/CGR)[3] and the follicle-stimulating hormone receptor (FSHR),[4] play a prominent role in human reproduction. The receptors, members of the GPCR superfamily of membrane proteins, are characterized by a large N-terminal ectodomain that binds the endogenous glycoprotein ligands. The endogenous ligands LH/hCG and FSH share an identical α-subunit and acquire their selectivity from their unique β-subunit. Recent developments in profertility research led to the discovery of several low molecular weight (LMW) agonists and antagonists for LH/CGR[5,6,7] and FSHR.[8-12] Since most of the described LMW ligands do not displace the radiolabeled endogenous glycoprotein it is assumed that the binding site is located in the transmembrane region of the receptors. It is of interest to note that some LMW ligands show dual activation of both the LHR and the FSHR. Chapter 5 describes that dimerization of one such LMW agonist showed altered pharmacological properties such as an increase in selectivity for the LHR and an increase in potency compared to the monomeric ligands.

Literature evidence reveals that receptor dimerization plays a role in signaling for both the LHR and the FSHR (see for a detailed discussion Chapter 1).[13-20] Probably the most direct evidence for gonadotropin receptor dimerization results from the recently resolved crystal structure of FSH, that is bound to the ectodomain of the receptor.[21,22] Here, the crystal complexes are organized in a dimeric fashion. The stability of the dimers is estimated to be rather weak, but is expected to be enhanced when the receptors are organized in their natural membrane bound state. Although the precise transmembrane interaction of the receptors can not be elucidated conclusively from the crystal data, it seems likely that transmembrane regions from two FSHR proteins are in close contact with each other.[22]

The aim of the present study is to establish to what extent dimerization of known LMW FSHR ligands will affect FSHR signaling. The tetrahydroquinoline (THQ, Figure 1) ligand **1** is a potent antagonist for the FSHR (IC_{50} 28 nM).[8] The (*R*)-enantiomer is the eutomer for the receptor while the (*S*)-enantiomer is inactive (distomer). Structure activity relationship studies for THQ **1** showed that small substituents on the phenyl ring are allowed without affecting the intrinsic antagonistic potencies of **1**. From these studies, it appears reasonable to assume that introduction of a propargyloxy group onto the phenyl substituent, as in **(*R*)-2**, would lead to a ligand that antagonizes the FSHR with a similar or only modestly reduced potency as **1**, and at the same time is amenable to dimerization with a number of bisazides to give dimeric ligands. In this Chapter the synthesis of such dimeric compounds is described. The ethylene glycol-based bisazides that also feature in Chapter 2 were selected as spacer entities. Further, both the (putative) active **(*R*)-2** and its inactive enantiomer **(*S*)-2** were prepared and included in the Huisgen [2+3]-cycloaddition as depicted in Figure 1. All compounds were evaluated on their FSHR-antagonizing potential.

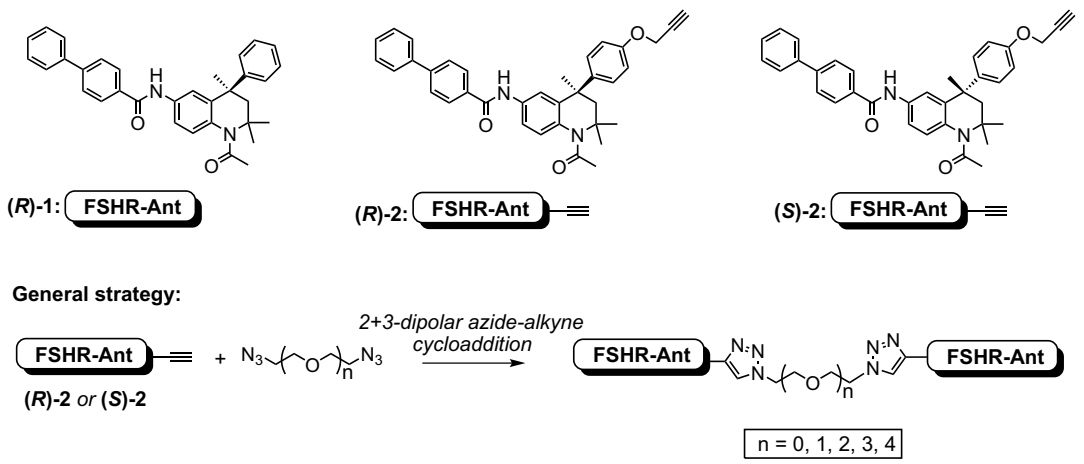
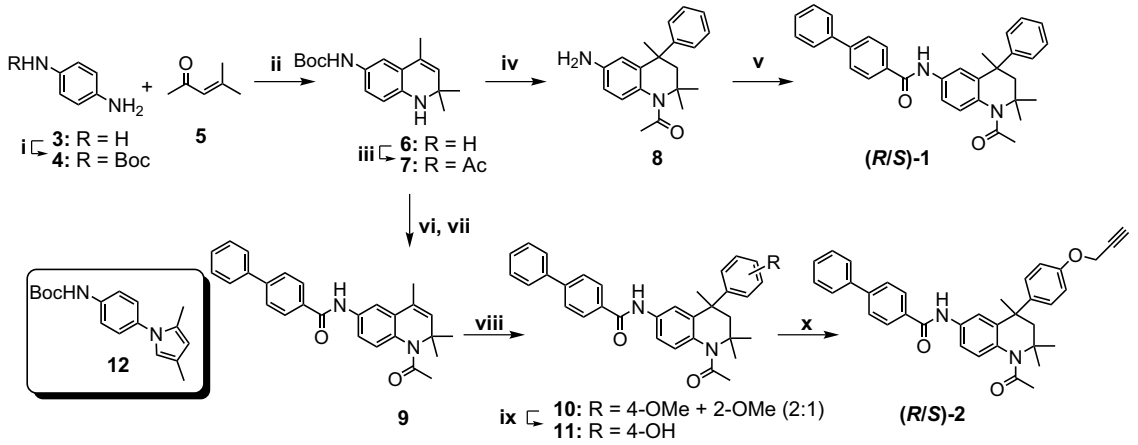


Figure 1. General strategy for the preparation of homodimeric FSHR ligands.

Results and discussion

The THQ **(*R*/*S*)-1** was prepared as described in literature and used as a reference compound in our library.[8] The acetylene functionalized racemic ligands **(*R*/*S*)-2** were synthesized with some adaptations of the described procedures, as depicted in Scheme 1. Thus, phenylenediamine (**3**)

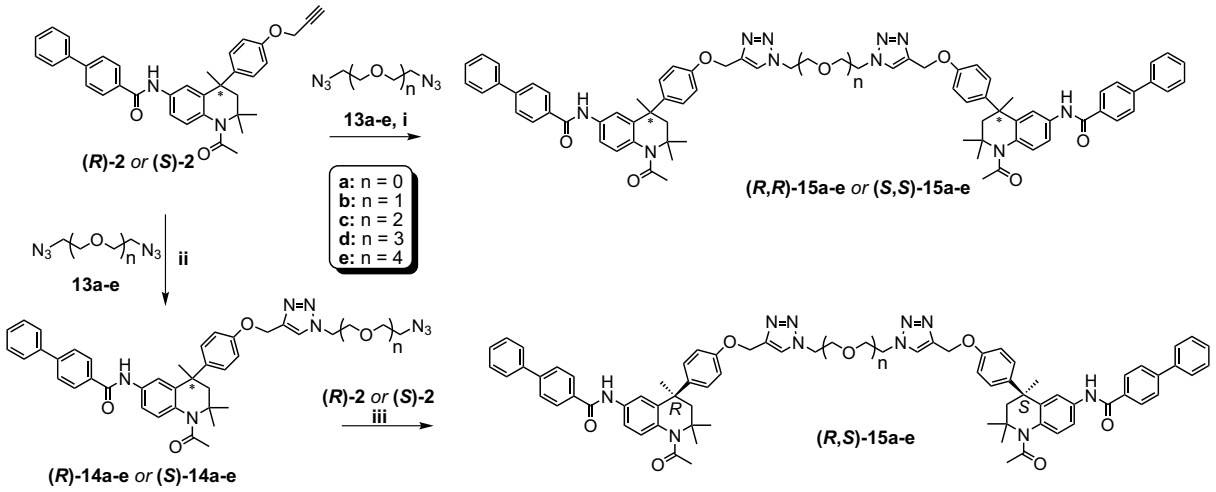
was subjected to 0.20 equivalents of di-*tert*-butyldicarbonate (Boc_2O) to afford mainly the mono-Boc protected amine **4**. Formation of dihydroquinoline **6** in a Skraup-Doebner-von Miller[23] reaction proved troublesome using the reported conditions[8] (mesityl oxide, I_2, acetone, 100 °C) and resulted in the formation of a significant amount of pyrrole **12**. The reaction was optimized by employment of acetone, $MgSO_4$, 4-*t*-butylcatechol (3.0 mol%) and iodine (5.0 mol%) as catalysts.[24] Although the conversion was not complete, the yield based on starting material was acceptable (74-96%). Regioselective acetylation of N-1 in **6** proceeded in 86% yield. Friedel-Crafts alkylation of benzene with compound **7** in the presence of aluminium chloride gave free amine **8** as a mixture of enantiomers in quantitative yield. Subsequent acylation of the 6-NH_2 with 4-phenylbenzoyl chloride gave the mixture of enatiomers **(*R*/*S*)-1** in 82% yield.



Scheme 1. Synthetic route for the synthesis of pharmacophore **(*R*/*S*)-1** and **(*R*/*S*)-2**. *Reagents and conditions*: *i.* Boc_2O, dioxane, 18 h, 76%; *ii.* acetone, I_2, 4-*t*-butylcatechol, $MgSO_4$, 2 days, 63 °C, 74-96% based on recovered starting material; *iii.* acetyl chloride/acetic anhydride, pyridine, DCM, 20 h, 86%. *iv*. $AlCl_3$, benzene, 30 min, 70 °C, quant; *v*. 4-phenylbenzoyl chloride, DiPEA, THF, 5 d, 82%; *vi*. 10% TFA in DCM, 5 h; *vii*. 4-phenylbenzoylchloride, DiPEA, THF, 4.5 h, 56% over two steps; *viii*. anisole, $AlCl_3$, 33-34.5 °C, 34 h, 54%, 2:1 mixture of 4-OMe and 2-OMe; *ix*. BBr_3, DCM, 0 °C , 12 h, 87%; *x*. propargyl bromide (80% in toluene), K_2CO_3, acetone, 2 d, 94%.

Friedel-Crafts alkylation of anisole with compound **7** proved to proceed sluggishly. Alternatively, the Boc-group in compound **7** was cleaved (TFA/DCM) and subsequent acylation of the free 6-aniline moiety with 4-phenylbenzoyl chloride gave compound **9** in 56% yield over the two steps. Friedel-Crafts alkylation of anisole with **9** provided *para* and *ortho* substituted compounds **10** (2:1 ratio) in 54% yield as a mixture of enantiomers. The methoxy compounds **10** were subjected to boron tribromide, which resulted in the exclusive cleavage of the 4-OMe without affecting the 2-OMe functionality in the mixture of compounds. At this stage the initially formed regioisomers were readily separated and compound **11** was then subjected to potassium carbonate and propargyl bromide to give target compound **2**, as a racemic mixture. The enantiomers were separated and purified by chiral preparative HPLC to yield the *R*-(+)- and *S*-(-)-quinolines **2** in 84% yield.

The enantiomerically pure ligands **(*R*)-2** and **(*S*)-2** were subsequently reacted in a Huisgen [2+3]-cycloaddition with a set of bis-azide spacers (Scheme 2). The homodimeric ligands **(*R*,*R*)-15a-e** and **(*S*,*S*)-15a-e** were prepared in high yields following a recently published method[25] that makes use of a biphasic solvent system (DCM/H_2O) and the presence of sodium ascorbate and copper sulfate. For the monomeric ligands, 0.2 equivalents of (*R*)- or (*S*)-acetylene **2** was subjected to the bis-azides **13a-e** in the presence of sodium ascorbate and copper sulfate. In this case, the biphasic solvent system provided somewhat lower yields. This was due to a significant amount of dimeric product formed probably due to the reactant distribution in the both phases of the solvent. The dimeric ligands **(*R*,*S*)-15a-e** were prepared in high yield by subjecting acetylene **(*S*)-2** to the monomeric azides **(*R*)-14a-e**.



Scheme 2. Synthesis of monomers, homodimers and heterodimers. *Reagents and conditions*: *i.* 0.5 eq **13a-e**, $CuSO_4$ (0.2 eq), sodium ascorbate (1 eq), DCM/H_2O, 50-100%; *ii.* 5 eq **13a-e**, $CuSO_4$ (0.2 eq), sodium ascorbate (1 eq), DCM/H_2O, 33-56%; *iii.* $CuSO_4$ (0.2 eq), sodium ascorbate (1 eq), DCM/H_2O, 88-100%.

The monomeric and dimeric ligands were then assayed on their antagonistic potencies on the FSH receptor. The IC_{50} values are the concentrations of the test compounds needed to inhibit recombinant FSH-stimulated CRE-luciferase activity in CHO-K1 cells expressing FSH receptors by 50%. The concentration of recombinant FSH is chosen to stimulate CRE-luciferase activity to 80% of the maximal attainable CRE-luciferase activity. The results are shown in Table 1. As expected, the monomeric compounds and homodimeric compounds that were derived from the (*S*)-enantiomer did not antagonize FSH mediated FSHR signaling up to 10 μM final concentration. Within the (*R*)-series, the potency for **(*R*)-2**, in which a propargyl function is located at the *para*-position of the phenyl ring, was in the same order of magnitude as compound **1**. The potency for the monomeric ligands **(*R*)-14a-e** was up to 20-fold lower than the compounds that lack the ethylene glycol moiety. The potency proportionally decreased with increasing spacer length. The antagonistic potencies for the homodimeric ligands **(*R*,*R*)-15a-e** as well as the heterodimeric ligands **(*R*,*S*)-15a-e** were significantly lower than the monomeric compounds **(*R*)-14a-e** ($P<0.05$). It appears that the antagonistic potencies of the heterodimeric

ligands **(*R*,*S*)-15a-e** decrease with increasing spacer length. The decrease in potency was also observed for the monomer compounds and the amount seems to be related to the spacer length in both series (depicted in Figure 2). The opposite trend is observed with homodimeric compounds **(*R*,*R*)-15a-e** that are derived from **(*R*)-2**. Here, the antagonistic potency increases with increasing spacer length. This observation may be the result of the second pharmacophore that plays a role in receptor binding when the spacer is of significant length. It should be noted that the differences in activities in the two series are minor, definitely too small to warrant definite conclusions. An interesting paradox emerges when comparing the different series of dimeric compounds **(*R*,*R*)-15a-e** and **(*R*,*S*)-15a-e** and their activities. One may expect that when the spacer length interconnecting two (*R*)-ligands is too short to allow ligand-binding to two individual receptors (for example, compound **(*R*,*R*)-15a**, with n=0), the potencies for this homodimeric ligand being similar (when the second active pharmacophore does not contribute to the potency) or enhanced (when the second active pharmacophore does contribute to the potency) when compared to the heterodimeric ligand **(*R*,*S*)-15a** (that has one inactive pharmacophore). However, the opposite is observed and homodimeric ligands **(*R*,*R*)-15a-b** with short spacer-length have a lower antagonistic potency than the heterodimeric ligands **(*R*,*S*)-15a-b** ($P<0.05$).

	Spacer (n)	IC_{50} FSHR (nM)		Spacer (n)	IC_{50} FSHR (nM)
		Monomeric ligands			
(*R*/*S*)-1	-	97			
(*R*)-2	-	39	**(*S*)-2**	-	n.a.
(*R*)-14a	n = 0	115	**(*S*)-14a**	n = 0	n.a.
(*R*)-14b	n = 1	141	**(*S*)-14b**	n = 1	n.a.
(*R*)-14c	n = 2	324	**(*S*)-14c**	n = 2	n.a.
(*R*)-14d	n = 3	379	**(*S*)-14d**	n = 3	n.a.
(*R*)-14e	n = 4	482	**(*S*)-14e**	n = 4	n.a.
		Homodimeric ligands			
(*R*,*R*)-15a	n = 0	>10000 *	**(*S*,*S*)-15a**	n = 0	n.a.
(*R*,*R*)-15b	n = 1	6725 *	**(*S*,*S*)-15b**	n = 1	n.a.
(*R*,*R*)-15c	n = 2	4712	**(*S*,*S*)-15c**	n = 2	n.a.
(*R*,*R*)-15d	n = 3	3712	**(*S*,*S*)-15d**	n = 3	n.a.
(*R*,*R*)-15e	n = 4	3107	**(*S*,*S*)-15e**	n = 4	n.a.
		Heterodimeric ligands			
(*R*,*S*)-15a	n = 0	2660			
(*R*,*S*)-15b	n = 1	2077			
(*R*,*S*)-15c	n = 2	7832 *			
(*R*,*S*)-15d	n = 3	9015 *			
(*R*,*S*)-15e	n = 4	>10000 *			

Table 1. Mean antagonistic potency (IC_{50}) of monomeric compounds and dimeric compounds on the FSH receptor. IC_{50} values are determined from two independent experiments performed in duplicate. SD of pIC_{50} is generally lower than 0.3. *: estimated IC_{50} due to an incomplete curve. n.a.: not active.

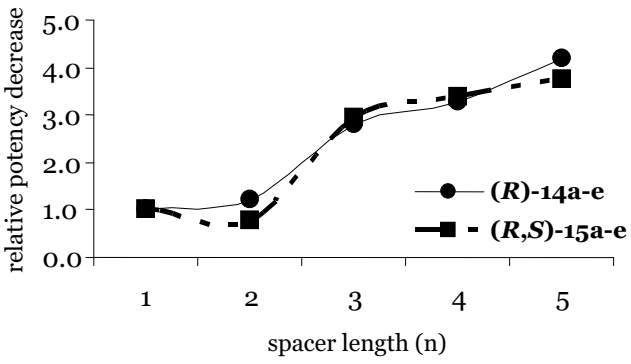


Figure 2. Relative potency decrease for monomeric compounds **(*R*)-14a-e** and heterodimeric compounds **(*R*,*S*)-15a-e**. The potency for the compound with n = 1 was set to 1.

It was recently described that FSH receptor dimerization is involved in FSHR signaling.[13,15,18,19] Evidence indicated that only one glycoprotein hormone binds to a receptor dimer and, when bound, the second binding site of the receptor-dimer becomes a low-affinity binding site.[13] This low-affinity binding site is only occupied when a high concentration of ligand is present. Binding of the ligand to this low-affinity binding site consequently results in a diminished binding affinity of the hormone to the initial high-affinity binding site, thereby circumventing overstimulation of the receptor at high hormone levels. This so-called negative cooperativity takes place in order to gain optimal sensitivity for hormones that are present at low concentrations and to maintain the activity level when a high concentration of hormone is present.[26] The antagonistic assays described in this Chapter are performed by measuring the potency of the compounds to inhibit 50% of the activity induced by a FSH concentration that effects 80% of the maximum stimulation (EC_{80}). The presence of the endogenous agonist FSH suggests that the receptors are present in a dimeric (or maybe even oligomeric) fashion. Two possible explanations may be given for the observed reverse trend in antagonistic potencies observed in the **(*R*,*R*)-15a-e** and **(*R*,*S*)-15a-e** dimeric series. The first explanation is based on the assumption that dimeric ligands **(*R*,*R*)-15a-e** with short spacers stabilize a receptor dimer, thereby enhancing the FSH signaling. This may seem unlikely since the length of the spacer between the recognition heads is very short, but if true the agonistic activity of these compounds may then be observed without the presence of recFSH. Additional assays in an agonistic set-up did not provide any evidence of allosteric agonism in any of the compounds. The second explanation is based on the assumption that the dimeric ligands **(*R*,*R*)-15a-e** induce a negative cooperativity effect. Upon binding of one ligand, the affinity for the second ligand is reduced. Dimeric ligands induce a high local concentration of the second ligand to the low-affinity binding site. Once the second ligand is bound, the affinity for the first ligand is diminished thus resulting in a decrease in antagonism (compared to heterodimeric ligands **(*R*,*S*)-15a-e**). Upon increasing spacer length, only one of the recognition units is bound to the receptor due to entropy reasons and the negative cooperativity effect is diminished (thus resulting in the increase in antagonistic potency). Chapter 2 details that modification and dimerization of a pharmacophore may lead to a decrease in antagonistic activity of the lead structure while the binding affinities are enhanced. It is difficult to establish whether

this is a result of ligand dimerization, whether the observed effect arises from tempering with a highly optimized structure or whether biophysical features (such as solubility or increased cell-membrane interactions) are involved. It may be that the results obtained in Chapter 2 also involve negative cooperativity, which would explain the unexpected trends in binding affinity and antagonist potency. It should be noted that additional experiments are needed to confirm this hypothesis.

Conclusion

The in this Chapter described results indicate the potential for dimeric ligands with enantiomeric properties, in which one active and one inactive enantiomer is present, as valuable tools to explore the mode of binding of dimeric ligands to the receptor (dimer). The dimeric ligands that incorporate two active enantiomers on either side of the spacer show an increase in activity upon increasing spacer length. When at one side of the linker an active ligand is replaced by an inactive enantiomer, the antagonistic potency decreases with increasing spacer length. This is also observed for the monomeric compounds. To further investigate the nature of the interaction of the dimeric ligands with the receptor, an additional set of bivalent ligands is needed. The next Chapter will address some of these questions by the incorporation of heterodimeric ligands consisting of an FSHR antagonist on one side and an FSHR agonist on the other.

Experimental procedures

Measurement of CRE-induced luciferase activity

Materials. Recombinant human LH (recLH) and human recombinant FSH (recFSH) were synthesized at Schering-Plough Research Institute, Oss, The Netherlands. Luclite® was obtained from Packard. All cell culture supplies were obtained from Gibco/BRL unless indicated otherwise. The human LH receptor cDNA[27] and human FSH receptor cDNA[28] were kindly provided by Dr. A.J.W. Hsueh, Stanford University.

Luciferase assay. Chinese Hamster Ovary (CHO)-K1 cells stably expressing the CRE-luciferase reporter with the human LH receptor or human FSH receptor were grown to 80-90% confluency in Dulbecco's MEM/Nutrient Mix F12 containing 5% bovine calf serum and supplemented with penicillin G (80 units/mL) and streptomycin (0.08 mg/mL) in 5% CO_2 at 37 °C. Cells were harvested using cell dissociation solution (Sigma). Aliquots of the cells were cryopreserved in DMSO without a loss of functional activity on LH receptor or FSH receptor.[29] On the day of the experiment, cells were thawed, washed with assay medium (Dulbecco's MEM/Nutrient Mix F12 supplemented with 1 mg/L bovine insulin (Sigma), 5 mg/L apo-transferrin (Sigma), penicillin G (80 units/mL) and streptomycin (0.08 mg/mL) and then resuspended in assay medium. The compounds were tested in quadruplicate at 10 concentrations ranging from final concentrations of 10 μM to 0.316 nM with half log intervals. In the agonistic assays, 10 μL of assay medium containing test compound and 3% DMSO, 10 μL of assay medium containing 3% DMSO with recLH (final concentration of 1 nM) or recFSH (final concentration of 586 pM) or 10 μL of assay medium containing 3% DMSO alone were added to the wells of a 384-well white culture plate followed by the

addition of 10 µL of assay medium. Then, 10 µL of cell suspension containing 7,500 cells was added to the wells. The final concentration of DMSO was 1%. In case of antagonistic assays, 10 µL of test compound solution or 10 µL of assay medium alone were added to 10 µL of assay medium containing recLH (final concentration of 100 pM, EC_{80}) or recFSH (final concentration of 49 pM, EC_{80}). Then, 10 µL of cell suspension containing 7,500 cells was added to the wells. After incubation for 4 h in a humidified atmosphere in 5% CO_2 at 37°C, plates were allowed to adjust to room temperature for 1 h. Then, 15 µL of LucLite solution (Packard) was added to the incubation mixture. Following 60 min at room temperature in the dark, luciferase activity was measured in a Packard Topcount Microplate Scintillation and Luminescence Counter. Agonistic effects of the compounds were determined as percentage of the (maximal) effect induced by 1 nM recLH or 586 pM recFSH. Antagonistic effects of the compounds were expressed as percentage of the effect induced by 100 pM recLH or 49 pM recFSH. The EC_{50} or IC_{50} values (concentration of the test compound that elicits half-maximal (50 %) luciferase stimulation or inhibition compared to the compound's maximally attainable effect, respectively) and the efficacy values (maximal effect as percentage of the effect of recLH or recFSH) of the test compounds were determined using the software program MathIQ (version 2.0, ID Business Solutions Limited).

Chemical procedures

Reactions were executed at ambient temperatures unless stated otherwise. All moisture sensitive reactions were performed under an argon atmosphere. All solvents were removed by evaporation under reduced pressure. Reactions were monitored by TLC analysis using silica gel coated plates (0.2 mm thickness) and detection by 254 nm UV-light or by either spraying with a solution of $(NH_4)_6Mo_7O_{24} \times 4H_2O$ (25 g/L) or $(NH_4)_4Ce(SO_4)_4 \times 2H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at ~150 °C. Column chromatography was performed on silica gel (40-63 µm). NMR spectra were recorded on a 200/50 MHz, 300/75 MHz, 400/100 MHz, 500/125 MHz or 600/150 MHz spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants (*J*) are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. Where indicated, NMR peak assignments were made using COSY, NOESY (τ mix = 1 sec) and HMQC experiments. For LC-MS analysis, a HPLC-system (detection simultaneously at 214 and 254 nm) equipped with an analytical C_{18} column (4.6 mmD × 250 mmL, 5µ particle size) in combination with buffers A: H_2O, B: CH_3CN and C: 1% aq TFA and coupled to a mass instrument with an electronspray interface (ESI) was used. For RP-HPLC purifications, an automated HPLC system equipped with a semi-preparative C_{18} column (5 µm C_{18}, 10Å, 150 × 21.2 mm) was used. The applied buffers were A: H_2O + ammonium acetate (20 mM) and B: CH_3CN. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60000 at *m/z* 400 (mass range *m/z* = 150-2000) and dioctylpthalate (*m/z* = 391.28428) as a lock mass. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). Optical rotations were measured on a Propol automatic polarimeter (Sodium D-line, λ = 589 nm). Optical rotations were measured on a Propol automatic polarimeter (Sodium D-line, λ = 589 nm). ATR-IR spectra were recorded on a Shimadzu FTIR-8300 fitted with a single bounce Durasample IR diamond crystal ATR-element and are reported in cm^{-1}.

***N*-Boc-1,4-phenylenediamine (4)**.To a solution of phenylenediamine (50.1 gram, 460 mmol, 5 eq) in dioxane (900 mL) on ice, a solution of Boc_2O (20.0 gram, 92 mmol) in dioxane (100 mL) was slowly added. The mixture was stirred overnight at rt. The solvent was evaporated, the remaining solid dissolved in DCM/MeOH (9/1) and washed with sat aq $NaHCO_3$, H_2O and brine. The organic layer was dried ($MgSO_4$), filtered and concentrated. Purification by column chromatography (20 to 50% EtOAc in PE) gave the desired product. Yield: 18.3 gram (88

mmol, 95%). R_f = 0.31 (50% EtOAc in PE). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.13 (d, 2H, CH Ar), 6.64 (d, 2H, CH Ar), 6.26 (s, 1 H, N*H*Boc), 3.06 (br s, 2H, NH_2), 1.50 (s, 9H, 3 × CH_3, Boc). ^{13}C NMR (MeOH, 400 MHz) δ 117.0 (4 × CH Ar), 28.8 (3 × CH_3, Boc). ESI-MS *m/z*: obsd 209.0 $[M + H]^+$.

6-(*tert*-Butoxycarbonyl)amino-1,2-dihydro-2,2,4-trimethylquinoline (6). Compound **4** (15.0 gram, 72 mmol) was dissolved in 170 mL acetone, $MgSO_4$ (43.35 gram, 0.361 mol, 5 eq) and *t*-butyl catechol (354 mg, 2.13 mmol, 3.0 mol%) were added. Iodine (915 mg, 3.60 mmol, 5.0 mol%) was added and the reaction was heated to reflux (63 °C) for 2 days. The mixture was diluted with EtOAc, filtrated and concentrated. The residue was dissolved in DCM and washed with 3M NaOH (4×) and water (3×) to remove most of the *t*-butyl catechol. The organic layer was dried ($MgSO_4$) and concentrated. The mixture was purified by silica gel column chromatography (50 to 100% toluene in PE) to obtain 8.0 gram (28 mmol, 39%, 96% based on recovered starting material) of a brown-yellow oil that became solid over time. R_f = 0.4 (11% EtOAc in toluene). ^{1}H NMR (200 MHz, MeOD) δ 7.67 (s, 1H, CH Ar), 7.19 – 7.04 (m, 2H, CH Ar), 6.52 (s, 1H, C=C*H*), 1.92 (s, 3H, CH_3(C=CH)), 1.49 (s, 9H, 3 × CH_3, Boc), 1.19 (s, 6H, 2 × CH_3). ^{13}C NMR (50 MHz, MeOD) δ 155.6 (C=O, Boc), 140.3, 135.3 (2 × C_q, Ar), 130.57 (C=*C*H), 129.6 (C_q, Ar), 123.6 (C_q, *C*=CH), 121.4, 116.8, 115.0 (3 × CH Ar), 80.4 (C_q, Boc), 52.7 (C_q), 30.4 (2 × CH_3), 29.2 (3 × CH_3, Boc), 14.9 (CH_3). ESI-MS *m/z*: 289.1 $[M + H]^+$. A side product *tert*-butyl-4-(2,4-dimethyl-1*H*-pyrrol-1-yl)phenylcarbamate (**14**) was isolated. R_f = 0.47 (10% EtOAc in PE). ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.47 – 7.38 (m, 2H, 2 × CH Ar), 7.21 – 7.11 (m, 2H, 2 × CH Ar), 6.57 – 6.50 (m, 2H, 1 × CH Ar, pyrrole + N*H*), 5.88 (s, 1H, CH Ar, pyrrole), 2.14 (s, 3H, CH_3), 2.01 (s, 3H, CH_3), 1.53 (s, 9H, 3 × CH_3, Boc). ESI-MS *m/z*: 287.1 $[M + H]^+$.

1-Acetyl -6-(*tert*-butoxycarbonyl)amino-1,2-dihydro-2,2,4-trimethylquinoline (7). A mixture of acetylchloride and acetic anhydride (4.54 mL, 1/1; v/v) was dropwise added to a solution of compound **6** (1.94 gram, 6.73 mmol) in DCM (50 mL) and pyridine (5 mL). After stirring for 20 h, the mixture was washed with 1.0 M HCl (3×) and water (3×) and the organic layer was dried ($MgSO_4$), filtered and concentrated *in vacuo*. Purification by silica gel column chromatography (0 to 10% EtOAc in toluene) gave compound **7** (1.9 gram, 5.94 mmol, 86%). R_f = 0.27 (11% EtOAc in toluene). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28 (s, 1H, CH Ar, DHQ-10), 7.17 (d, *J* = 8.0, 1H, CH Ar, DHQ-8), 6.78 (d, *J* = 8.4, 1H, CH Ar, DHQ-7), 5.54 (s, 1H, C=C*H*), 2.13 (s, 3H, CH_3), 2.02 (s, 3H, CH_3, acetyl), 1.52 (2 × s, 15 H, 5 × CH_3, Boc + C(CH_3)$_2$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.9 (C=O, acetyl), 152.9 (*C*=O, Boc), 136.8 (*C*H=C), 135.21 (C_q Ar), 132.1 (C_q, CH=*C*), 129.9 (C_q Ar), 127.6 (C_q Ar), 124.3 (CH Ar, DHQ-7), 117.0 (CH Ar, DHQ-8), 113.4 (CH Ar, DHQ-10), 80.5 (C_q, Boc), 58.0 (C_q, C(CH_3)$_2$), 28.3 (CH_3, Boc), 26.5 (CH_3), 25.9 (CH_3, acetyl), 17.8 (CH_3). ESI-MS *m/z*: obsd 331.0 $[M + H]^+$.

1-Acetyl -6-amino-4-phenyl-1,2,3,4-tetrahydro-2,2,4-trimethylquinoline (8). A suspension of $AlCl_3$ (0.643 gram, 4.8 mmol, 10 eq) in benzene (5 mL) was added to a solution of compound **7** (0.160 gram, 0.48 mmol) in benzene (5 mL). Additional $AlCl_3$ was added (10 eq) and the reaction mixture was stirred at 70 °C for 30 minutes. The mixture was cooled to 0 °C, quenched with water and washed with 2M NaOH. The organic layer was dried ($MgSO_4$) and concentrated. Silica gel column chromatography (0 to 14% EtOAc in toluene) yielded compound **8** (150 mg, 0.48 mmol, 100%). R_f = 0.1 (16% EtOAc in toluene). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.22 – 7.19 (m, 2H, 2 × CH Ar), 7.15 – 7.11 (m, 3H, 3 × CH Ar), 6.75 (d, *J* = 8.5, 1H, CH Ar, THQ-7), 6.71 (s, 1H, CH Ar, THQ-10), 6.59 (dd, *J* = 8, 2.5, 2.5, 1H, CH Ar, THQ-8), 3.74 (br s, 2H, NH_2), 2.52 (d, *J* = 13.5, 1H, CH_2, THQ), 1.89 (s, 3H, CH_3, acetyl), 1.76 (d, *J* = 13.5, 1H, CH_2, THQ), 1.58 (s, 3H, CH_3), 1.31 (s, 3H, CH_3), 1.25 (s, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.8 (C=O, acetyl), 145.2 (C_q, Ar, THQ-5), 144.1 (C_q, Ar, THQ-6), 142.3 (C_q, Ar, phenyl-16), 130.9 (C_q, Ar, THQ-9), 127.9 (2 × CH Ar), 127.3 (CH Ar, THQ-7), 127.2, 126.2 (3 × CH Ar), 112.9 (CH Ar, THQ-8), 112.3 (CH Ar, THQ-10), 58.2 (C_q Ar, THQ-4), 56.1 (CH_2, THQ-3), 42.4 (C_q, C(CH_3)$_2$), 29.6 (CH_3), 29.2 (CH_3), 28.8 (CH_3), 25.3 (CH_3, acetyl). ESI-MS *m/z*: obsd 309.2 $[M + H]^+$.

1-Acetyl-4-phenyl-6-(4-phenylbenzoyl)amino-1,2,3,4-tetrahydro-2,2,4-trimethylquinoline (R/S)-1. Compound **8** (50 mg, 0.165 mmol) was dissolved in 5 mL THF and DiPEA (110 µL, 0.66 mmol, 4 eq) and 4-phenylbenzoylchloride (80 mg, 0.35 mmol, 2 eq) were added. After 20 h of stirring another equivalent of 4-phenylbenzoylchloride (40 mg, 0.165 mmol) was added together with an equivalent of DiPEA (28 µL) and again after 22 and 40 h of stirring. TLC showed completion of the reaction after 48 h. The mixture was concentrated and the residue dissolved in EtOAc and washed with 0.5 M HCl, water, 5% aq $NaHCO_3$, water and brine. The organic layer was dried ($MgSO_4$), and concentrated. Purification of the residue by silica gel column chromatography (0 to 10% EtOAc in toluene) gave the desired compound **(R/S)-1** (66 mg, 0.135 mmol) as a white solid in 82% yield. R_f = 0.5 (33% EtOAc in toluene). ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.06 (s, 1H, N*H*CO), 7.96 (d, *J* = 8, 2H, 2 × CH Ar), 7.72 (d, *J* = 8.5, 1H, CH Ar, THQ-8), 7.71 (s, 1H, CH Ar, THQ-10), 7.59 – 7.55 (m, 4H, 4 × CH Ar), 7.46 – 7.37 (m, 3H, 3 × CH Ar), 7.17 – 7.06 (m, 5H, 5 × CH Ar), 6.83 (d, *J* = 8, 1H, CH Ar, THQ-7), 2.51 (d, *J* = 14, 1H, CH_2, THQ), 1.86 (s, 3H, CH_3, acetyl), 1.75 (d, *J* = 14, 1H, CH_2, THQ), 1.56 (s, 3H, CH_3), 1.31 (s, 3H, CH_3), 1.21 (s, 3H, CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.2 (C=O, acetyl), 165.9 (C=O, amide), 144.7, 144.4, 141.5, 139.7, 136.0, 135.5, 133.4 (7 × C_q Ar), 128.8 (2 × CH Ar), 128.1 (CH Ar), 127.9 (2 × CH Ar), 127.8 (2 × CH Ar), 127.1 (4 × CH Ar), 126.8 (2 × CH Ar), 126.4 (CH Ar), 126.2 (CH Ar, THQ-7), 118.7 (CH Ar, THQ-8), 117.7 (CH Ar, THQ-10), 58.4 (C_q, THQ-4), 55.9 (CH_2, THQ-3), 42.4 (C_q, $C(CH_3)_2$), 29.6 (CH_3), 29.3 (CH_3), 28.6 (CH_3), 25.3 (CH_3, acetyl). HRMS *m/z* calcd for $C_{40}H_{42}N_8O_4 + H^+$: 489.25365, obsd 489.25327.

1-Acetyl-4-phenyl-6-(4-phenylbenzoyl)amino-1,2-dihydro-2,2,4-trimethylquinoline (9). Compound **7** (25.1 gram, 0.076 mol) was dissolved in DCM (250 mL) and a mixture of DCM (250 mL) and TFA (75 mL) was added (total concentration of 15% TFA in DCM). The reaction was stirred for 5h and toluene was added. The mixture was concentrated, dissolved in DCM (250 mL) and washed with sat aq $NaHCO_3$ (2 × 100 mL). The organic layer was dried ($MgSO_4$), concentrated and co-evaporated with toluene (3×). NMR of the crude product showed removal of the Boc protecting group. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 7.30 – 7.28 (m, 1H, CH Ar), 6.91 – 6.81 (m, 2H, 2 × CH Ar), 6.39 (br s, 2H, NH_2), 5.54 (s, 1H, C=CH), 2.06 (s, 3H, CH_3), 1.96 (s, 3H, CH_3), 1.49 (s, 6H, 2 × CH_3). The crude product was dissolved in dry THF (500 mL). DiPEA (120 mL, 0.69 mol, 9 eq) and 4-phenylbenzoylchloride (32.6 gram, 0.15 mmol, 2 eq) were added and the mixture was stirred for 4.5 h at room temperature. After evaporation of the solvent, the residue was dissolved in EtOAc and washed with 1,0 M HCl (3×), H_2O (2×), 2M NaOH (2×), H_2O, brine. The product was isolated by silica gel column chromatography (0 to 10% EtOAc in toluene) to afford compound **9** (17.5 gram, 0.043 mol) as white crystals in 56% yield. R_f = 0.26 (20% EtOAc in toluene). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.40 (s, 1H, NHCO), 7.96 (d, *J* = 8.4, 2H, CH Ar), 7.66 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.63 – 7.61 (m, 2H, 2 × CH Ar), 7.59 (s, 1H, CH Ar, DHQ-10), 7.49 – 7.45 (m, 3H, 3 × CH Ar), 7.41 – 7.37 (m, 1H, CH Ar), 6.81 (d, *J* = 8.4, CH Ar, DHQ-7), 5.55 (s, 1H, C=C*H*), 2.13 (s, 3H, CH_3, acetyl), 2.02 (s, 3H, CH_3), 1.52 (s, 6H, 2 × CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.1 ((C=O, acetyl), 165.6 (C=O, amide), 144.6, 139.7 (2 × C_q Ar), 136.8 (C=*C*H), 134.8 (C_q Ar), 133.3 (C_q, *C*=CH), 133.3, 129.8 (2 × C_q Ar, DHQ), 128.9 (2 × CH Ar), 128.1 (CH Ar), 127.6 (2 × CH Ar), 127.5 (C_q Ar, DHQ), 127.3 (2 × CH Ar), 127.1 (2 × CH Ar), 124.1 (CH Ar, DHQ-7), 118.8 (CH Ar, DHQ-8), 115.2 (CH Ar, DHQ-10), 58.1 (C_q, $C(CH_3)_2$), 26.5 (2 × CH_3), 26.0 (CH_3, acetyl), 17.8 (CH_3). ESIMS *m/z*: obsd 411.3 $[M + H]^+$.

1-Acetyl-4-(4-methoxypheny)-6-(4-phenylbenzoyl)amino-1,2,3,4-tetrahydro-2,2,4trimethyl-quinoline and 1-Acetyl-4-(2-methoxypheny)-6-(4-phenylbenzoyl)amino-1,2,3,4tetrahydro-2,2,4-trimethylquinoline (10). To a solution of compound **9** (2.24 gram, 5.46 mmol) in 100 mL anisole $AlCl_3$ (2.18 gram, 16.37 mmol, 3 eq) was added and the mixture was stirred at 33 °C for 16 h. TLC showed that the reaction was not complete, so another equivalent of $AlCl_3$ (0.73 gram, 5.46 mmol) was added and the temperature was raised to 34.5 °C. After 18 h the reaction was still not completed, but TLC showed the formation of unwanted by-

products, so the reaction was cooled (0 °C), quenched with H_2O, washed with 2M NaOH, dried ($MgSO_4$) and concentrated. The residue was purified by silica gel column chromatography (0 to 40% EtOAc in PE). This resulted in pure compound **10** (1.51 gram, 2.9 mmol) as a fine white powder in 54% yield as a 2:1 mixture of 4-methoxyphenyl and 2-methoxyphenyl products. R_f = 0.55 (50% EtOAc in PE).

4-OMe: ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.96 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.95 (s, 1H, N*H*CO), 7.72 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.69 (d, *J* = 8.4, 1H, CH Ar, THQ-8), 7.63 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.61 (s, 1H, CH Ar, THQ-10), 7.48 (t, *J* = 7.2, 7.8, 2H, 2 × CH Ar), 7.41 (t, *J* = 7.2, 7.2, CH Ar), 7.02 (d, *J* = 9, 2H, 2 × CH Ar), 6.97 (d, *J* = 8.4, 1H, CH Ar, THQ-7), 6.75 (d, *J* = 9, 2H, 2 × CH Ar), 3.76 (s, 3H, OC*H*$_3$), 2.51 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.96 (s, 3H, C*H*$_3$, acetyl), 1.79 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.64 (s, 3H, C*H*$_3$(C-CH$_2$)), 1.36 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$), 1.26 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (C=O, acetyl), 165.4 (C=O, amide), 157.9 (*C*$_q$(OMe)), 144.8, 142.0, 139.7, 136.8, 136.0, 135.3, 133.3 (7 × C$_q$ Ar), 128.9 (2 × CH Ar), 128.1 (CH Ar), 127.9 (2 × CH Ar), 127.5, 127.5 (4 × CH Ar), 127.2 (2 × CH Ar), 126.7 (CH Ar, THQ-7), 118.5 (CH Ar, THQ-8), 117.3 (CH Ar, THQ-10), 113.2 (2 × CH Ar), 58.5 (C$_q$, THQ-4), 56.1 (*C*H$_2$, THQ-3), 55.1 (CH$_3$, OCH$_3$), 41.9 (C$_q$, *C*(CH$_3$)$_2$), 29.7 (CH$_3$), 29.3 (CH$_3$), 28.7 (CH$_3$), 25.5 (CH$_3$, acetyl). ESI-MS *m/z*: obsd 519.5 $[M + H]^+$.

2-OMe: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.56 (s, 1H, N*H*CO), 7.96 (d, *J* = 8.0, 2H, 2 × CH Ar), 7.71 (d, *J* = 8.8, 1H, CH Ar), 7.67 – 7.58 (m, 4H, 4 × CH Ar), 7.48 – 7.39 (m, 3H, 3 × CH Ar), 7.26 – 7.11 (m, 5H, 5 × CH Ar), 6.89 – 6.77 (m, 2H, 2 × CH Ar), 6.63 – 6.54 (m, 2H, 2 × CH Ar), 3.86 (s, 3H, OCH$_3$), 3.16 (d, *J* = 13.6, 1H, CH$_2$), 1.90 (s, 3H, CH$_3$, acetyl), 1.70 (s, 3H, CH$_3$), 1.48 (d, *J* = 13.6, 1H, CH$_2$), 1.33 (s, 3H, CH$_3$), 1.21 (s, 3H, CH$_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.3 (C=O, acetyl), 165.8 (C=O, amide), 158.7 (*C*$_q$(OMe)), 144.6, 143.7, 139.9, 137.9, 135.7, 133.6, 132.2 (7 × C$_q$ Ar), 129.1, 129.0, 128.8, 128.3, 128.2, 128.1, 127.4, 127.7, 126.7, 125.3, 124.2, 120.2, 118.6, 117.5, 111.1 (16 × CH Ar), 58.6 (C$_q$, THQ-4), 54.7 (CH$_3$, OCH$_3$), 52.2 (CH$_2$, THQ-3), 42.7 (C$_q$, *C*(CH$_3$)$_2$), 29.2, 26.7, 25.6, 25.0 (4 × CH$_3$). ESI-MS *m/z*: obsd 519.7 $[M + H]^+$.

1-Acetyl-4-(4-hydroxypheny)l-6-(4-phenylbenzoyl)amino-1,2,3,4-tetrahydro-2,2,4trimethyl-quinoline (11). Boron tribromide (1M in DCM, 15.5 mL, 14.65 mmol, 5 eq) was slowly added to a cooled (0 °C) solution of compound **10** (2.29 g, 4.40 mmol, mixture of 2-OMe and 4-OMe) in DCM (150 mL). The mixture was gently warmed up to room temperature and stirred for 12 h. TLC showed completion of the reaction. The mixture was poured into water, EtOAc was added and the organic layer was washed with sat aq $NaHCO_3$ (2×) and brine. The organic layer was dried ($MgSO_4$), concentrated and the residue was purified by silica gel column chromatography (0 to 33% EtOAc in toluene). Pure compound **11** (1.29 g, 2.55 mmol) was obtained as a fine white powder in 87% yield. R_f = 0.39 (50% EtOAc in PE). ^{1}H NMR (600 MHz, MeOD) δ 8.04 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.85 (s, 1H, CH Ar, THQ-10), 7.78 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.74 (d, *J* = 8.4, 1H, CH Ar, THQ-8), 7.69 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.48 (t, *J* = 7.8, 2H, 2 × CH Ar), 7.39 (t, *J* = 7.2, 1H, CH Ar), 7.06 (d, *J* = 8.4, 1H, CH Ar, THQ-7), 6.97 (d, *J* = 9, 2H, 2 × CH Ar), 6.65 (d, *J* = 8.4, 2H, 2 × CH Ar), 4.60 (br s, 1H, OH), 2.61 (d, *J* = 13.8, 1H, CH$_2$, THQ), 1.96 (s, 3H, CH$_3$, acetyl), 1.75 (d, *J* = 13.8, 1H, CH$_2$, THQ), 1.63 (s, 3H, CH$_3$), 1.32 (s, 3H, CH$_3$), 1.25 (s, 3H, CH$_3$). ^{13}C NMR (150 MHz, MeOD) δ 173.6 (C=O, acetyl), 168.6 (C=O, amide), 156.9 (C$_q$OH), 146.0, 143.6, 141.2, 138.0, 137.0, 136.7, 134.8 (7 × C$_q$ Ar), 130.1 (2 × CH Ar), 129.3 (4 × CH Ar), 129.2 (CH Ar), 128.1, 128.1 (4 × CH Ar), 127.9 (CH Ar, THQ-7), 120.4 (CH Ar, THQ-8), 119.4 (CH Ar, THQ-10), 115.7 (2 × CH Ar), 60.1 (C$_q$, THQ-4), 57.0 (CH$_2$, THQ-3), 43.1 (C$_q$, C(CH$_3$)$_2$), 30.4 (CH$_3$), 29.7 (CH$_3$), 29.2 (CH$_3$), 25.7 (CH$_3$, acetyl).

1-Acetyl-4-(4-(prop-2-yn-1-yloxy)pheny)l-6-(4-phenylbenzoyl)amino-1,2,3,4-tetrahydro-2,2,4-trimethylquinoline (*R*/*S*-2). Potassium carbonate (409 mg, 2.80 mmol, 1.1 eq) was added to a solution of compound **11** (1.29 gram, 2.55 mmol) in acetone (250 mL) and the mixture was refluxed for 30 minutes at 65 °C. Propargyl bromide (80% in toluene, 0.36 mL, 3.32 mmol, 1.3 eq) was added and the mixture was refluxed for 2 days until TLC showed completion of the reaction. The mixture was concentrated, the residue dissolved in EtOAc,

washed with water (3×) and brine. The organic layer was dried ($MgSO_4$), concentrated and purified by silica gel column chromatography (0 to 20% EtOAc in toluene). Compound **(*R*/*S*-2)** (1.30 gram, 2.40 mmol) was obtained in 94% yield as a white crystalline compound. Chiral separation of compound **(*R*/*S*-2)** (1.2 gram, 2.2 mmol) on a chiral IB column (15% EtOH in heptane) gave pure enantiomers in 84% yield. (*R*-isomer: 555 mg, 1.02 mmol, *S*-isomer: 457 mg, 0.84 mmol). R_f = 0.56 (50% EtOAc in PE).

(+)-*R*-2: ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.01 (s, 1H, NHCO), 7.96 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.72 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.67 (d, *J* = 8.4, 1H, CH Ar, THQ-8), 7.63 (d, *J* = 6.6, 3H, 3 × CH Ar), 7.48 (t, *J* = 7.2, 7.8, 2H, 2 × CH Ar), 7.41 (t, *J* = 7.2, 7.2, 1H, CH Ar), 7.03 (d, *J* = 9, 2H, 2 × CH Ar), 6.96 (d, *J* = 8.4, 1H, CH Ar, THQ-7), 6.83 (d, *J* = 8.4, 2H, 2 × CH Ar), 4.64 (s, 2H, OCH_2), 2.55-2.49 (m, 2H, CH_2 THQ + CH propargyl), 1.95 (s, 3H, CH_3, acetyl), 1.81 (d, *J* = 14.4, 1H, CH_2, THQ), 1.64 (s, 3H, $CH_3(C\text{-}CH_2)$), 1.36 (s, 3H, CH_3, $C(CH_3)_2$), 1.26 (s, 3H, CH_3, $C(CH_3)_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.2 (C=O, acetyl), 165.4 (C=O, amide), 155.9 ($C_q(OCH_2R)$), 144.8, 141.8, 139.7, 137.8, 136.0, 135.3, 133.3 (7 × C_q Ar), 128.9 (CH Ar), 128.1 (CH Ar), 128.0 (2 × CH Ar), 127.5, 127.4 (4 × CH Ar), 127.2 (2 × CH Ar), 126.7 (CH Ar, THQ-7), 118.5 (CH Ar, THQ-8), 117.4 (CH Ar, THQ-10), 114.2 (2 × CH Ar), 78.5, 75.5 (C_q +CH, $C_q{\equiv}CH$), 58.5 (C_q, THQ-4), 56.1 (CH_2, THQ-3), 55.8 (CH_2, OCH_2R), 41.9 (C_q, $C(CH_3)_2$), 29.7 (CH_3), 29.3 (CH_3), 28.7 (CH_3), 25.4 (CH_3, acetyl). $[\alpha]_D^{20}$: +375° (c=0.19, $CHCl_3$). ESI-MS *m/z*: obsd 543.6 $[M + H]^+$. HRMS *m/z* calcd for $C_{36}H_{34}N_2O_3 + H^+$: 543.26422, obsd 543.26422.

(-)-*S*-2: ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.98 (s, 1H, NHCO), 7.96 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.72 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.67 (dd, *J* = 8.4, 1.8, 1H, CH Ar, THQ-8), 7.63 (d, *J* = 7.2, 3H, 3 × CH Ar), 7.48 (t, *J* = 7.5, 2H, 2 × CH Ar), 7.41 (t, *J* = 7.8, 7.2, 1H, CH Ar), 7.03 (d, *J* = 8.4, 2H, 2 × CH Ar), 6.97 (d, *J* = 8.4, 1H, CH Ar, THQ-7), 6.83 (d, *J* = 8.4, 2H, 2 × CH Ar), 4.64 (s, 2H, OCH_2), 2.52-2.49 (m, 2H, CH_2 THQ + CH propargyl), 1.95 (s, 3H, CH_3, acetyl), 1.80 (d, *J* = 13.8, 1H, CH_2, THQ), 1.64 (s, 3H, CH_3), 1.36 (s, 3H, CH_3), 1.26 (s, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.2 (C=O, acetyl), 165.4 (C=O, amide), 156.0 ($C_q(OCH_2R)$), 144.8, 141.9, 139.7, 137.8, 136.0, 135.3, 133.3 (7 × C_q Ar), 129.0 (CH Ar), 128.2 (CH Ar), 128.0 (2 × CH Ar), 127.5, 127.5 (4 × CH Ar), 127.2 (2 × CH Ar), 126.7 (CH Ar, THQ-7), 118.6 (CH Ar, THQ-8), 117.4 (CH Ar, THQ-10), 114.3 (2 × CH Ar), 78.5, 75.5 (C_q + CH, $C_q{\equiv}CH$), 58.5 (C_q, THQ-4), 56.1 (CH_2, THQ-3), 55.8 (CH_2, OCH_2R), 41.9 (C_q, $C(CH_3)_2$), 29.7 (CH_3), 29.4 (CH_3), 28.7 (CH_3), 25.4 (CH_3, acetyl). $[\alpha]_D^{20}$: -410° (c=0.17, $CHCl_3$). ESI-MS *m/z*: obsd 543.6 $[M + H]^+$. HRMS *m/z* calcd for $C_{36}H_{34}N_2O_3 + H^+$: 543.26422, obsd 543.26410.

General procedure for the preparation of monomeric ligands (R)-14a-e and (S)-14a-e.

Compound **(*R*)-2** or **(*S*)-2** (50 mg, 0.092 mmol, 1 eq) was dissolved in DCM (10 mL) and PEG-diazide spacer **13a**, **13b**, **13c**, **13d** or **13e** (n=0-4, 0.92 mmol, 10 eq) was added. The mixture was degassed under argon in an ultrasonic bath, until the volume was approximately 1.5 mL. Water and solutions of $CuSO_4$ and sodium ascorbate were degassed separately. Water (1.0 mL) and aqueous solutions of sodium ascorbate (50 µL, 0.092 mmol, 1 eq) and $CuSO_4$ (50 µL, 0.018 mmol, 20 mol%) were added. After stirring for 24 h, TLC and LC-MS showed most reactions to be completed. Additional amounts of $CuSO_4$ and sodium ascorbate were added if the reaction was not finished yet. After 48 h all reactions were finished. Reaction mixtures were concentrated and coevaporated with toluene (3×). The residue was dissolved in DCM and the product was isolated using silica column chromatography (0 to 1% MeOH in DCM for monomer, 1 to 2.5% MeOH in DCM for dimer). Pure products were obtained as white powdery crystals in 39-53% yield. Also dimer formation was observed, which was purified as well and obtained in 25-38% yield. So the overall yield of the reaction ranged between 75-84%. R_f = 0.60 (9% MeOH in DCM).

Monomeric ligand (*R*)-14a. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.96 (d, *J* = 7.8, 3H, 2 × CH Ar + NHCO), 7.72 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.67 – 7.63 (m, 5H, 5 × CH Ar), 7.48 (t, *J* = 7.5, 2H, 2 × CH Ar), 7.41 (t, *J* = 7.2, 1H, 1 × CH Ar), 7.03 (d, *J* = 8.4, 2H, 2 × CH Ar), 6.98 (d, *J* = 8.4, 1H, 1 × CH Ar, THQ-7), 6.84 (d, *J* = 8.4, 2H, 2 × CH Ar), 5.19 (s, 2H, CH_2, OCH_2Ctrz), 4.49 (t, *J* = 5.4, 6.0, 2H, CH_2, CH_2Ntrz), 3.82 (t, *J* = 5.4, 6.0, 2H, CH_2, CH_2N_3), 2.50

(d, *J* = 13.8, 1H, CH_2, THQ), 1.95 (s, 3H, CH_3, acetyl), 1.80 (d, *J* = 13.8, 1H, CH_2, THQ), 1.58 (s, 3H, CH_3), 1.35 (s, 3H, CH_3), 1.25 (s, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (C=O, acetyl), 165.4 (C=O, amide), 156.5 ($C_q(OCH_2Trz)$), 144.8, 144.5, 141.9, 139.7, 137.5, 136.0, 135.4, 133.3 (8 × C_q Ar), 129.0 (2 × CH Ar), 128.2 (CH Ar), 128.1 (2 × CH Ar), 127.5, 127.5, 127.2 (6 × CH Ar), 126.7 (CH Ar, THQ-7), 123.5 (CH Trz), 118.5 (CH Ar, THQ-8), 117.4 (CH Ar, THQ-10), 114.3 (2 × CH Ar), 62.0 (CH_2, OCH_2Ctrz), 58.5 (C_q, THQ-4), 56.2 (CH_2, THQ-3), 50.6 (CH_2, CH_2N_3), 49.4 (CH_2, CH_2Ntrz), 41.9 (C_q, $C(CH_3)_2$), 29.4 (2 × CH_3), 28.7 (CH_3), 25.5 (CH_3, acetyl). $[\alpha]_D^{20}$: +291° (c=0.094, $CHCl_3$). ESI-MS *m/z*: obsd 655.27 $[M + H]^+$. HRMS *m/z* calcd for $C_{38}H_{38}N_8O_3$+ H^+: 655.31396, obsd 655.31396.

Monomeric ligand (*R*)-14b. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.40 (s, 1H, NHCO), 7.98 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.82 (br s, 1H, CH Ar), 7.70 (t, *J* = 6.0, 8.4, 3H, 3 × CH Ar), 7.66 (s, 1H, CH Ar, THQ-10), 7.62 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.48 (t, *J* = 7.8, 7.8, 2H, 2 × CH Ar), 7.40 (t, *J* = 7.8, 7.2, 1H, 1 × CH Ar), 7.01 (d, *J* = 8.4, 2H, 2 × CH Ar), 6.94 (d, *J* = 9.0, 1H, 1 × CH Ar, THQ-7), 6.84 (d, *J* = 8.4, 2H, 2 × CH Ar), 5.14 (s, 2H, CH_2, OCH_2Ctrz), 4.56 (s, 2H, CH_2, CH_2Ntrz), 3.86 (t, *J* = 4.8, 4.2, 2H, CH_2, $NtrzCH_2CH_2O$), 3.59 (t, *J* = 4.8, 4.8, 2H, CH_2, $OCH_2CH_2N_3$), 3.33 (t, *J* = 4.8, 2H, CH_2, CH_2N_3), 2.49 (d, *J* = 13.8, 1H, CH_2, THQ), 1.94 (s, 3H, CH_3, acetyl), 1.78 (d, *J* = 13.8, 1H, CH_2, THQ), 1.59 (s, 3H, CH_3), 1.35 (s, 3H, CH_3), 1.24 (s, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.2 (C=O, acetyl), 165.6 (C=O, amide), 156.5 ($C_q(OCH_2Trz)$), 144.6, 141.8, 139.7, 137.4, 135.8, 135.6, 133.3 (7 × C_q Ar), 128.9 (2 × CH Ar), 128.1 (CH Ar), 128.0 (2 × CH Ar), 127.6, 127.3, 127.1 (6 × CH Ar), 126.6 (CH Ar, THQ-7), 120.5 (CH Trz), 118.6 (CH Ar, THQ-8), 117.5 (CH Ar, THQ-10), 114.2 (2 × CH Ar), 70.0 (CH_2, $OCH_2CH_2N_3$), 69.3 (CH_2, $NtrzCH_2CH_2O$), 61.8 (CH_2, OCH_2Ctrz), 58.5 (C_q, THQ-4), 56.1 (CH_2, THQ-3), 50.7, 50.5 (2 × CH_2, CH_2N_3 + CH_2Ntrz), 41.8 (C_q, $C(CH_3)_2$), 29.6 (CH_3), 29.3 (CH_3), 28.7 (CH_3), 25.4 (CH_3, acetyl). $[\alpha]_D^{20}$: +260° (c=0.05, $CHCl_3$). ESI-MS *m/z*: obsd 699.33 $[M + H]^+$. HRMS *m/z* calcd for $C_{40}H_{42}N_8O_4$ + H^+: 699.34018, obsd 699.34039.

Monomeric ligand (*R*)-14c. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.33 (s, 1H, NHCO), 7.98 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.81 (br s, 1H, CH Trz), 7.70 (d, *J* = 8.4, 3H, 3 × CH Ar), 7.65 (s, 1H, CHar, THQ-10), 7.63 (d, *J* = 7.2, 2H, 2 × CH Ar), 7.48 (t, *J* = 7.2, 7.8, 2H, 2 × CH Ar), 7.41 (t, *J* = 7.8, 7.2, 1H, 1 × CH Ar), 7.02 (d, *J* = 8.4, 2H, 2 × CH Ar), 6.96 (d, *J* = 8.4, 1H, 1 × CH Ar, THQ-7), 6.84 (d, *J* = 8.4, 2H, 2 × CH Ar), 5.14 (s, 2H, CH_2, OCH_2Ctrz), 4.54 (t, *J* = 4.8, 4.8, 2H, CH_2, CH_2Ntrz), 3.87 (t, *J* = 5.4, 4.8, 2H, CH_2, $NtrzCH_2CH_2O$), 3.62 (t, *J* = 4.8, 5.4, 6H, 3 × CH_2, OCH_2CH_2O + $OCH_2CH_2N_3$), 3.35 (t, *J* = 5.4, 4.8, 2H, CH_2, CH_2N_3), 2.49 (d, *J* = 14.4, 1H, CH_2, THQ), 1.95 (s, 3H, CH_3, acetyl), 1.79 (d, *J* = 13.8, 1H, CH_2, THQ), 1.62 (s, 3H, CH_3), 1.36 (s, 3H, CH_3), 1.25 (s, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (C=O, acetyl), 165.5 (C=O, amide), 156.6 ($C_q(OCH_2Trz)$), 144.6, 141.8, 139.7, 137.4, 135.8, 135.5, 133.3 (7 × C_q Ar), 128.9 (2 × CH Ar), 128.4 (CH Ar), 128.0 (2 × CH Ar), 127.6, 127.6, 127.1 (6 × CH Ar), 126.6 (CH Ar, THQ-7), 120.5 (CH Trz), 118.6 (CH Ar, THQ-8), 117.5 (CH Ar, THQ-10), 114.1 (2 × CH Ar), 70.5, 70.4, 70.0, 69.4 (4 × CH_2, OCH_2CH_2O + $OCH_2CH_2N_3$ + $NtrzCH_2CH_2O$), 61.9 (CH_2, OCH_2Ctrz), 58.4 (C_q, THQ-4), 56.1 (CH_2, THQ-3), 50.5, 50.3 (2 × CH_2, CH_2N_3 + CH_2Ntrz), 41.8 (C_q, $C(CH_3)_2$), 29.6 (CH), 29.3 (CH_3), 28.7 (CH_3), 25.3 (CH_3, acetyl). $[\alpha]_D^{20}$: +243° (c=0.028, $CHCl_3$). ESI-MS *m/z*: obsd 743.40 $[M + H]^+$. HRMS *m/z* calcd for $C_{42}H_{46}N_8O_5$ + H^+: 743.36639, obsd 743.36652.

Monomeric ligand (*R*)-14d. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.29 (s, 1H, NHCO), 7.97 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.69 (d, *J* = 8.4, 3H, 3 × CH Ar), 7.63 (s, 1H, CH Ar, THQ-10), 7.62 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.47 (t, *J* = 7.2, 7.8, 2H, 2 × CH Ar), 7.39 (t, *J* = 7.2, 1H, 1 × CH Ar), 7.01 (d, *J* = 7.8, 2H, 2 × CH Ar), 6.94 (d, *J* = 8.4, 1H, 1 × CH Ar, THQ-7), 6.84 (s, 2H, 2 × CH Ar), 5.12 (s, 2H, CH_2, OCH_2Ctrz), 4.54 (s, 2H, CH_2, CH_2Ntrz), 3.86 (s, 2H, CH_2, $NtrzCH_2CH_2O$), 3.66-3.59 (m, 10H, 5 × CH_2, OCH_2CH_2O + $OCH_2CH_2N_3$), 3.33 (t, *J* = 4.8, 4.8, 2H, CH_2, CH_2N_3), 2.48 (d, *J* = 13.8, 1H, CH_2, THQ), 1.94 (s, 3H, CH_3, acetyl), 1.78 (d, *J* = 13.8, 1H, CH_2, THQ), 1.61 (s, 3H, CH_3), 1.34 (s, 3H, CH_3), 1.24 (s, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.2 (C=O, acetyl), 165.5 (C=O, amide), 156.6

($C_q(OCH_2Trz)$), 144.7, 141.8, 139.7, 137.4, 135.8, 135.5, 133.4 (7 × C_q Ar), 128.9 (2 × CH Ar), 128.1 (CH Ar), 128.0 (2 × CH Ar), 127.6, 127.4, 127.2 (6 × CH Ar), 126.6 (CH Ar, THQ-7), 118.6 (CH Ar, THQ-8), 117.5 (CH Ar, THQ-10), 114.2 (2 × CH Ar), 70.6, 70.5, 70.49, 70.0, 69.2 (6 × CH_2, OCH_2CH_2O + $OCH_2CH_2N_3$ + $NtrzCH_2CH_2O$), 61.8 (CH_2, OCH_2Ctrz), 58.5 (C_q, THQ-4), 56.1 (CH_2, THQ-3), 50.6 (2 × CH_2, CH_2N_3 + CH_2Ntrz), 41.9 (C_q, $C(CH_3)_2$), 29.6 (CH_3), 29.3 (CH_3), 28.7 (CH_3), 25.5 (CH_3, acetyl). $[\alpha]_D^{20}$: +229° (c=0.096, $CHCl_3$). ESI-MS *m/z*: obsd 787.40 $[M + H]^+$. HRMS *m/z* calcd for $C_{44}H_{50}N_8O_6 + H^+$: 787.39261, obsd 787.39264.

Monomeric ligand (*R*)-14e. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.37 (s, 1H, NHCO), 7.97 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.71 – 7.67 (m, 3H, 3 × CH Ar), 7.63 (s, 1H, CH Ar, THQ-10), 7.61 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.46 (t, *J* = 7.8, 2H, 2 × CH Ar), 7.39 (t, *J* = 7.2, 7.8, 1H, 1 × CH Ar), 7.00 (d, *J* = 7.8, 2H, 2 × CH Ar), 6.93 (d, *J* = 8.4, 1H, 1 × CH Ar, THQ-7), 6.82 (d, *J* = 7.8, 2H, 2 × CH Ar), 5.12 (s, 2H, CH_2, OCH_2Ctrz), 4.52 (s, 2H, CH_2, CH_2Ntrz), 3.84 (t, *J* = 4.8, 4.2, 2H, CH_2, $NtrzCH_2CH_2O$), 3.62-3.57 (m, 14H, 7 × CH_2, OCH_2CH_2O + $OCH_2CH_2N_3$), 3.33 (t, *J* = 4.8, 5.4, 2H, CH_2, CH_2N_3), 2.47 (d, *J* = 13.8, 1H, CH_2, THQ), 1.93 (s, 3H, CH_3, acetyl), 1.77 (d, *J* = 13.8, 1H, CH_2, THQ), 1.60 (s, 3H, CH_3), 1.34 (s, 3H, CH_3), 1.24 (s, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (C=O, acetyl), 165.5 (C=O, amide), 156.6 ($C_q(OCH_2Trz)$), 144.6, 141.8, 139.7, 137.4, 135.8, 135.6, 133.4 (7 × C_q Ar), 128.9 (2 × CH Ar), 128.4 (CH Ar), 128.0 (2 × CH Ar), 127.64, 127.3, 127.1 (6 × CH Ar), 126.6 (CH Ar, THQ-7), 118.6 (CH Ar, THQ-8), 117.5 (CH Ar, THQ-10), 114.1 (2 × CH Ar), 70.6, 70.6, 70.5, 70.4, 69.9, 69.3 (8 × CH_2, OCH_2CH_2O + $OCH_2CH_2N_3$ + $NtrzCH_2CH_2O$), 61.8 (CH_2, OCH_2Ctrz), 58.4 (C_q, THQ-4), 56.1 (CH_2, THQ-3), 50.6, 50.4 (2 × CH_2, CH_2N_3 + CH_2Ntrz), 41.8 (C_q, $C(CH_3)_2$), 29.6 (CH_3), 29.3 (CH_3), 28.7 (CH_3), 25.5 (CH_3, acetyl). $[\alpha]_D^{20}$: +266° (c=0.23, $CHCl_3$). ESI-MS *m/z*: obsd 831.47 $[M + H]^+$. HRMS *m/z* calcd for $C_{46}H_{54}N_8O_7 + H^+$: 831.41882, obsd 831.41962.

Monomeric ligand (*S*)-14a. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.18 (s, 1H, N*H*CO), 7.96 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.70 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.68 – 7.61 (m, 5H, 5 × CH Ar), 7.47 (t, *J* = 7.5, 2H, 2 × CH Ar), 7.40 (t, *J* = 7.5, 1H, 1 × CH Ar), 7.01 (d, *J* = 8.4, 2H, 2 × CH Ar), 6.95 (d, *J* = 8.4, 1H, 1 × CH Ar, THQ-7), 6.83 (d, *J* = 9.0, 2H, 2 × CH Ar), 5.17 (s, 2H, C*H*$_2$, OC*H*$_2$Ctrz), 4.48 (t, *J* = 5.4, 6.0, 2H, C*H*$_2$, C*H*$_2$Ntrz), 3.80 (t, *J* = 6.0, 5.4, 2H, C*H*$_2$, C*H*$_2$N$_3$), 2.49 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.93 (s, 3H, CH_3, acetyl), 1.78 (d, *J* = 14.4, 1H, C*H*$_2$, THQ), 1.62 (s, 3H, CH_3), 1.34 (s, 3H, CH_3), 1.24 (s, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (*C*=O, acetyl), 165.5 (*C*=O, amide), 156.5 (*C*$_q$(OCH$_2$Trz)), 144.7, 144.4, 141.8, 139.7, 137.5, 135.8, 135.5, 133.3 (8 × C_q Ar), 128.9 (2 × CH Ar), 128.1 (CH Ar), 128.0 (2 × CH Ar), 127.6, 127.4, 127.1 (6 × CH Ar), 126.6 (CH Ar, THQ-7), 123.5 (CH Trz), 118.6 (CH Ar, THQ-8), 117.4 (CH Ar, THQ-10), 114.2 (2 × CH Ar), 61.9 (*C*H$_2$, OC*H*$_2$Ctrz), 58.5 (C_q, THQ-4), 56.1 (*C*H$_2$, THQ-3), 50.6 (*C*H$_2$, *C*H$_2$N$_3$), 49.4 (*C*H$_2$, *C*H$_2$Ntrz), 41.9 (C_q, *C*(CH$_3$)$_2$), 29.6 (CH_3), 29.3 (CH_3), 28.7 (CH_3), 25.4 (*C*H$_3$, acetyl). $[\alpha]_D^{20}$: 298° (c=0.11, $CHCl_3$). ESI-MS *m/z*: obsd 655.33 $[M + H]^+$. HRMS *m/z* calcd for $C_{38}H_{38}N_8O_3$+ H^+: 655.31396, obsd 655.31390.

Monomeric ligand (*S*)-14b. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.40 (s, 1H, N*H*CO), 7.99 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.72 – 7.70 (m, 4H, 4 × CH Ar), 7.68 (s, 1H, CH Ar, THQ-10), 7.63 (d, *J* = 7.2, 2H, 2 × CH Ar), 7.48 (t, *J* = 7.2, 7.8, 2H, 2 × CH Ar), 7.41 (t, *J* = 7.2, 1H, 1 × CH Ar), 7.02 (d, *J* = 9.0, 2H, 2 × CH Ar), 6.96 (d, *J* = 8.4, 1H, 1 × CH Ar, THQ-7), 6.85 (d, *J* = 9.0, 2H, 2 × CH Ar), 5.16 (s, 2H, C*H*$_2$, OC*H*$_2$Ctrz), 4.56 (t, *J* = 4.8, 4.8, 2H, C*H*$_2$, C*H*$_2$Ntrz), 3.87 (t, *J* = 5.4, 4.8, 2H, C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.60 (t, *J* = 4.8, 5.4, 2H, C*H*$_2$, OC*H*$_2$CH$_2$N$_3$), 3.33 (t, *J* = 4.8, 2H, C*H*$_2$, C*H*$_2$N$_3$), 2.50 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.95 (s, 3H, CH_3, acetyl), 1.79 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.63 (s, 3H, CH_3), 1.35 (s, 3H, CH_3), 1.24 (s, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (*C*=O, acetyl), 165.5 (*C*=O, amide), 156.6 (*C*$_q$(OCH$_2$Trz)), 144.6, 141.7, 139.8, 137.4, 135.8, 135.6, 133.4 (7 × C_q Ar), 128.9 (2 × CH Ar), 128.0 (CH Ar), 128.0 (2 × CH Ar), 127.6, 127.23, 127.1 (6 × CH Ar), 126.6 (CH Ar, THQ-7), 118.5 (CH Ar, THQ-8), 117.4 (CH Ar, THQ-10), 114.2 (2 × CH Ar), 70.1 (*C*H$_2$, OC*H*$_2$CH$_2$N$_3$), 69.4 (*C*H$_2$, NtrzCH$_2$*C*H$_2$O), 61.8 (*C*H$_2$, OC*H*$_2$Ctrz), 58.4 (C_q, THQ-4), 56.1 (*C*H$_2$, THQ-3), 50.5, 50.3 (2 × *C*H$_2$, *C*H$_2$N$_3$ + *C*H$_2$Ntrz), 41.8 (C_q, *C*(CH$_3$)$_2$), 29.6

(CH_3), 29.3 (CH_3), 28.6 (CH_3), 25.4 (CH_3, acetyl). $[\alpha]_D^{20}$: -269° (c=0.10, CHCl3). ESI-MS *m/z*: obsd 699.40 [M + H]+. HRMS *m/z* calcd for $C_{40}H_{42}N_8O_4 + H^+$: 699.34018, obsd 699.34021.

Monomeric ligand (*S*)-14c. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.05 (s, 1H, N*H*CO), 7.93 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.75 (s, 1H, CH Trz), 7.68 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.65 (dd, *J* = 8.4, 2.4, 1H, CH Ar, THQ-8), 7.60 (d, *J* = 7.2, 3H, 3 × CH Ar), 7.45 (t, *J* = 7.2, 7.8, 2H, 2 × CH Ar), 7.37 (t, *J* = 7.8, 7.2, 1H, 1 × CH Ar), 6.99 (d, *J* = 9.0, 2H, 2 × CH Ar), 6.93 (d, *J* = 8.4, 1H, 1 × CH Ar, THQ-7), 6.81 (d, *J* = 8.4, 2H, 2 × CH Ar), 5.13 (s, 2H, C*H*$_2$, OC*H*$_2$Ctrz), 4.51 (t, *J* = 4.8, 5.4, 2H, C*H*$_2$, C*H*$_2$Ntrz), 3.84 (t, *J* = 5.4, 4.8, 2H, C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.58 (t, *J* = 5.4, 5.4, 6H, 3 × C*H*$_2$, OC*H*$_2$C*H*$_2$O + OC*H*$_2$CH$_2$N$_3$), 3.31 (t, *J* = 4.8, 4.8, 2H, C*H*$_2$, C*H*$_2$N$_3$), 2.46 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.95 (s, 3H, CH_3, acetyl), 1.79 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.62 (s, 3H, CH_3), 1.36 (s, 3H, CH_3), 1.25 (s, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.2 (*C*=O, acetyl), 165.5 (*C*=O, amide), 156.7 (*C*$_q$(OCH$_2$Trz)), 144.8, 144.0, 141.9, 139.8, 137.4, 136.0, 135.4, 133.4 (8 × C_q Ar), 129.0 (2 × CH Ar), 128.2 (CH Ar), 128.1 (2 × CH Ar), 127.6, 127.5, 127.2 (6 × CH Ar), 126.7 (CH Ar, THQ-7), 124.0 (CH Trz), 118.6 (CH Ar, THQ-8), 117.4 (CH Ar, THQ-10), 114.2 (2 × CH Ar), 70.5, 70.5, 70.1, 69.4 (4 × *C*H$_2$, OC*H*$_2$C*H*$_2$O + OC*H*$_2$CH$_2$N$_3$ + NtrzCH$_2$C*H*$_2$O), 62.0 (*C*H$_2$, OC*H*$_2$Ctrz), 58.5 (C_q, THQ-4), 56.2 (*C*H$_2$, THQ-3), 50.6, 50.32 (2 × *C*H$_2$, *C*H$_2$N$_3$ + *C*H$_2$Ntrz), 41.9 (C_q, *C*(CH$_3$)$_2$), 29.6 (CH_3), 29.4 (CH_3), 28.7 (CH_3), 25.5 (CH_3, acetyl). $[\alpha]_D^{20}$: -300° (c=0.10, $CHCl_3$). ESI-MS *m/z*: obsd 743.40 $[M + H]^+$. HRMS *m/z* calcd for $C_{42}H_{46}N_8O_5 + H^+$: 743.36639, obsd 743.36646.

Monomeric ligand (*S*)-14d. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.12 (s, 1H, N*H*CO), 7.98 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.84 (br s, 1H, CH Trz), 7.73 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.71 (dd, *J* = 8.4, 2.4, 1H, CH Ar, THQ-8), 7.64 (d, *J* = 7.8, 3H, 3 × CH Ar), 7.49 (t, *J* = 7.2, 7.8, 2H, 2 × CH Ar), 7.42 (t, *J* = 7.8, 7.2, 1H, 1 × CH Ar), 7.03 (d, *J* = 8.4, 2H, 2 × CH Ar), 6.97 (d, *J* = 8.4, 1H, 1 × CH Ar, THQ-7), 6.86 (d, *J* = 9.0, 2H, 2 × CH Ar), 5.16 (s, 2H, C*H*$_2$, OC*H*$_2$Ctrz), 4.55 (t, *J* = 4.8, 4.8, 2H, C*H*$_2$, C*H*$_2$Ntrz), 3.88 (t, *J* = 4.8, 4.8, 2H, C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.64-3.62 (m, 10H, 5 × C*H*$_2$, OC*H*$_2$C*H*$_2$O + OC*H*$_2$CH$_2$N$_3$), 3.36 (t, *J* = 4.8, 4.8, 2H, C*H*$_2$, C*H*$_2$N$_3$), 2.51 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.97 (s, 3H, CH_3, acetyl), 1.81 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.61 (s, 3H, CH_3), 1.37 (s, 3H, CH_3), 1.26 (s, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (*C*=O, acetyl), 165.4 (*C*=O, amide), 156.6 (*C*$_q$(OCH$_2$Trz)), 144.7, 141.9, 139.7, 137.4, 135.9, 135.4, 133.3 (7 × C_q Ar), 128.9 (2 × CH Ar), 128.1 (CH Ar), 128.0 (2 × CH Ar), 127.6, 127.4, 127.2 (6 × CH Ar), 126.6 (CH Ar, THQ-7), 118.6 (CH Ar, THQ-8), 117.4 (CH Ar, THQ-10), 114.1 (2 × CH Ar), 70.6, 70.5, 70.5, 70.0, 69.3 (6 × *C*H$_2$, OC*H*$_2$C*H*$_2$O + OC*H*$_2$CH$_2$N$_3$ + NtrzCH$_2$C*H*$_2$O), 61.9 (*C*H$_2$, OC*H*$_2$Ctrz), 58.5 (C_q, THQ-4), 56.1 (*C*H$_2$, THQ-3), 50.6, 50.3 (2 × *C*H$_2$, *C*H$_2$N$_3$ + *C*H$_2$Ntrz), 41.9 (C_q, *C*(CH$_3$)$_2$), 29.6 (CH_3), 29.3 (CH_3), 28.7 (CH_3), 25.5 (CH_3, acetyl). $[\alpha]_D^{20}$: -271° (c=0.096, $CHCl_3$). ESI-MS *m/z*: obsd 787.47 $[M + H]^+$. HRMS *m/z* calcd for $C_{44}H_{50}N_8O_6 + H^+$: 787.39261, obsd 787.39288.

Monomeric ligand (*S*)-14e. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.17 (s, 1H, N*H*CO), 7.95 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.81 (s, 1H, CH Trz), 7.73 – 7.70 (m, 3H, 3 × CH Ar), 7.64 (d, *J* = 7.2, 3H, 3 × CH Ar), 7.49 (t, *J* = 7.2, 7.8, 2H, 2 × CH Ar), 7.42 (t, *J* = 7.8, 7.2, 1H, 1 × CH Ar), 7.03 (d, *J* = 8.4, 2H, 2 × CH Ar), 6.97 (d, *J* = 8.4, 1H, 1 × CH Ar, THQ-7), 6.85 (d, *J* = 9.0, 2H, 2 × CH Ar), 5.16 (s, 2H, C*H*$_2$, OC*H*$_2$Ctrz), 4.54 (t, *J* = 5.4, 4.8, 2H, C*H*$_2$, C*H*$_2$Ntrz), 3.87 (t, *J* = 4.8, 4.8, 2H, C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.65 – 3.60 (m, 14H, 7 × C*H*$_2$, OC*H*$_2$C*H*$_2$O + OC*H*$_2$CH$_2$N$_3$), 3.36 (t, *J* = 4.8, 5.4, 2H, C*H*$_2$, C*H*$_2$N$_3$), 2.50 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.97 (s, 3H, CH_3, acetyl), 1.80 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.61 (s, 3H, CH_3), 1.37 (s, 3H, CH_3), 1.26 (s, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (*C*=O, acetyl), 165.4 (*C*=O, amide), 156.6 (*C*$_q$(OCH$_2$Trz)), 144.7, 143.8, 141.8, 139.7, 137.4, 135.9, 135.4, 133.3 (8 × C_q Ar), 128.9 (2 × CH Ar), 128.1 (CH Ar), 128.0 (2 × CH Ar), 127.6, 127.4, 127.1 (6 × CH Ar), 126.6 (CH Ar, THQ-7), 124.0 (CH Trz), 118.5 (CH Ar, THQ-8), 117.4 (CH Ar, THQ-10), 114.2 (2 × CH Ar), 70.6, 70.6, 70.5, 70.4, 69.9, 69.3 (8 × *C*H$_2$, OC*H*$_2$C*H*$_2$O + OC*H*$_2$CH$_2$N$_3$ + NtrzCH$_2$C*H*$_2$O), 61.9 (*C*H$_2$, OC*H*$_2$Ctrz), 58.44 (C_q, THQ-4), 56.1 (*C*H$_2$, THQ-3), 50.6, 50.3 (2 × *C*H$_2$, *C*H$_2$N$_3$ + *C*H$_2$Ntrz), 41.9 (C_q, *C*(CH$_3$)$_2$), 29.6 (CH_3), 29.3 (CH_3), 28.7 (CH_3), 25.4 (CH_3, acetyl).

$[\alpha]_D^{20}$: -287° (c=0.14, $CHCl_3$). ESI-MS *m/z*: obsd 831.47 $[M + H]^+$. HRMS *m/z* calcd for $C_{46}H_{54}N_8O_7 + H^+$: 831.41882, obsd 831.41901.

General procedure for the preparation of dimeric ligands (*R,R*)-15a-e and (*S,S*)-15a-e.
Compound **(*R*)-2** or **(*S*)-2** (30 mg, 0.055 mmol, 5 eq) was dissolved in DCM (10 mL) and PEG-diazide spacer **13a**, **13b**, **13c**, **13d** or **13e** (n=0-4, 0.022 mmol, 2 eq) was added. The mixture was degassed under argon in an ultrasonic bath, until the volume was approximately 1.5 mL. Water and solutions of $CuSO_4$ and sodium ascorbate were degassed separately. Water (1.0 mL) and aqueous solutions of sodium ascorbate (50 µL, 0.055 mmol, 5 eq) and $CuSO_4$ (50 µL, 0.011 mmol, 20 mol%) were added. After stirring for 24 h, TLC and LC-MS showed most reactions to be completed. Additional amounts of $CuSO_4$ and sodium ascorbate were added if the reaction was not finished yet. After 48 h all reactions were finished. Reaction mixtures were concentrated and coevaporated with toluene (3×). The residue was dissolved in DCM and the product was isolated using silica column chromatography (1 to 2.5% MeOH in DCM for dimer). Pure products were obtained as white powdery crystals in 50-100% yield. R_f = 0.45 (9% MeOH in DCM).

Dimeric ligand (*R,R*)-15a. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.44 (s, 2H, 2 × N*H*CO), 7.96 (d, *J* = 7.8, 4H, 4 × CH Ar), 7.68 (d, *J* = 8.4, 6H, 6 × CH Ar), 7.66 (s, 2H, CH Ar), 7.62 (d, *J* = 7.2, 4H, 4 × CH Ar), 7.48 (t, *J* = 7.2, 7.8, 4H, 4 × CH Ar), 7.41 (t, *J* = 7.2, 7.2, 2H, 2 × CH Ar), 6.98 (d, *J* = 9.0, 4H, 4 × CH Ar), 6.92 (d, *J* = 8.4, 2H, 2 × CH Ar, THQ-7), 6.76 (d, *J* = 8.4, 4H, 4 × CH Ar), 5.09 – 5.03 (m, 4H, 2 × C*H*$_2$, OC*H*$_2$Ctrz), 4.81 (s, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 2.47 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.89 (s, 6H, 2 × CH_3, acetyl), 1.77 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.61 (s, 6H, 2 × CH_3), 1.34 (s, 6H, 2 × CH_3), 1.26 (s, 6H, 2 × CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (2 × *C*=O, acetyl), 165.6 (2 × *C*=O, amide), 156.3 (2 × C_q(OCH$_2$Trz)), 144.6, 141.8, 139.7, 137.6, 135.7, 135.6, 133.3 (14 × C_q Ar), 128.9 (4 × CH Ar), 128.1 (2 × CH Ar), 128.0 (4 × CH Ar), 127.6, 127.3, 127.1 (12 × CH Ar), 126.6 (2 × CH Ar, THQ-7), 118.6 (2 × CH Ar, THQ-8), 117.5 (2 × CH Ar, THQ-10), 114.2 (4 × CH Ar), 61.6 (2 × *C*H$_2$, OC*H*$_2$Ctrz), 58.5 (2 × C_q, THQ-4), 56.1 (2 × *C*H$_2$, THQ-3), 49.5 (2 × *C*H$_2$, *C*H$_2$Ntrz), 41.9 (2 × C_q, *C*(CH$_3$)$_2$), 29.5 (2 × CH_3), 29.3 (2 × CH_3), 28.8 (2 × CH_3), 25.4 (2 × CH_3). $[\alpha]_D^{20}$: +334° (c=0.26, $CHCl_3$). ESI-MS *m/z*: obsd 1197.53 $[M + H]^+$. HRMS *m/z* calcd $C_{74}H_{72}N_{10}O_6 + H^+$: 1197.57091, obsd 1197.57275, calcd for $C_{74}H_{72}N_{10}O_6 + 2H^+$: 599.28909, obsd 599.28908.

Dimeric ligand (*R,R*)-15b. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.62 (s, 2H, 2 × N*H*CO), 7.96 (d, *J* = 8.4, 4H, 4 × CH Ar), 7.71 (dd, *J* = 8.4, 2.4, 2H, 2 × CH Ar), 7.66 (d, *J* = 8.4, 4H, 4 × CH Ar), 7.62 (s, 2H, 2 × CH Ar, THQ-10), 7.60 (d, *J* = 7.2, 4H, 4 × CH Ar), 7.49 (br s, 2H, 2 × CH Trz), 7.47 (t, *J* = 7.8, 4H, 4 × CH Ar), 7.40 (t, *J* = 7.2, 7.8, 2H, 2 × CH Ar), 6.95 (d, *J* = 8.4, 4H, 4 × CH Ar), 6.89 (d, *J* = 9.0, 2H, 2 × CH Ar, THQ-7), 6.76 (d, *J* = 8.4, 4H, 4 × CH Ar), 5.09 (s, 4H, 2 × C*H*$_2$, OC*H*$_2$Ctrz), 4.46-4.41 (m, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 3.76 (t, *J* = 4.8, 4H, 2 × C*H*$_2$, C*H*$_2$OC*H*$_2$), 2.45 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.89 (s, 6H, 2 × CH_3, acetyl), 1.75 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.60 (s, 6H, 2 × CH_3), 1.34 (s, 6H, 2 × CH_3), 1.21 (s, 6H, 2 × CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.2 (2 × *C*=O, acetyl), 165.7 (2 × *C*=O, amide), 156.5 (2 × C_q(OCH$_2$Trz)), 144.6, 141.7, 139.8, 137.5, 135.7, 135.7, 133.4 (14 × C_q Ar), 129.0 (4 × CH Ar), 128.1 (2 × CH Ar), 128.0 (4 × CH Ar), 127.7, 127.3, 127.2 (12 × CH Ar), 126.6 (2 × CH Ar, THQ-7), 125.3 (2 × CH Trz), 118.7 (2 × CH Ar, THQ-8), 117.6 (2 × CH Ar, THQ-10), 114.2 (4 × CH Ar), 69.2 (2 × *C*H$_2$, *C*H$_2$OCH$_2$), 61.7 (2 × *C*H$_2$, OC*H*$_2$Ctrz), 58.5 (2 × C_q, THQ-4), 56.1 (2 × *C*H$_2$, THQ-3), 50.1 (2 × *C*H$_2$, *C*H$_2$Ntrz), 41.8 (2 × C_q, *C*(CH$_3$)$_2$), 29.6 (2 × CH_3), 29.3 (2 × CH_3), 28.7 (2 × CH_3), 25.5 (2 × CH_3, acetyl). $[\alpha]_D^{20}$: +318 ° (c=0.32, $CHCl_3$). ESI-MS *m/z*: obsd 1241.47 $[M + H]^+$. HRMS *m/z* calcd $C_{76}H_{76}N_{10}O_7 + H^+$: 1241.59712, obsd 1241.59879, calcd for $C_{76}H_{76}N_{10}O_7 + 2H^+$: 621.30220, obsd 621.30200.

Dimeric ligand (*R*,*R*)-15c. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.72 (s, 2H, 2 × N*H*CO), 7.99 (d, *J* = 7.8, 4H, 4 × CH Ar), 7.76 (d, *J* = 7.2, 4H, 4 × CH Ar), 7.67 (d, *J* = 8.4, 4H, 4 × CH Ar), 7.61 (d, *J* = 7.2, 6H, 6 × CH Ar), 7.47 (t, *J* = 7.8, 7.8, 4H, 4 × CH Ar), 7.40 (t, *J* = 7.2, 7.8, 2H, 2 × CH Ar), 6.97 (d, *J* = 8.4, 4H, 4 × CH Ar), 6.91 (d, *J* = 8.4, 2H, 2 × CH Ar, THQ-7), 6.78 (d, *J* = 8.4, 4H, 4 × CH Ar), 5.10 (s, 4H, 2 × C*H*$_2$, OC*H*$_2$Ctrz), 4.45 (s, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 3.76 (d, *J* = 4.8, 4H, 2 × C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.47 (s, 4H, 2 × C*H*$_2$, OC*H*$_2$C*H*$_2$O), 2.45 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.91 (s, 6H, 2 × CH_3, acetyl), 1.76 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.56 (s, 6H, 2 × CH_3), 1.35 (s, 6H, CH_3), 1.23 (s, 6H, 2 × CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (2 × *C*=O, acetyl), 165.7 (2 × *C*=O, amide), 156.5 (2 × *C*$_q$(OCH$_2$Trz)), 144.5, 141.7, 139.7, 137.5, 135.7, 135.6, 133.4 (14 × C_q Ar), 128.9 (4 × CH Ar), 128.1 (2 × CH Ar), 128.0 (4 × CH Ar), 127.8, 127.2, 127.1 (12 × CH Ar), 126.5 (2 × CH Ar, THQ-7), 118.7 (2 × CH Ar, THQ-8), 117.6 (2 × CH Ar, THQ-10), 114.1 (4 × CH Ar), 70.3 (2 × *C*H$_2$, OC*H*$_2$*C*H$_2$O), 69.2 (2 × *C*H$_2$, NtrzCH$_2$*C*H$_2$O), 61.8 (2 × *C*H$_2$, OC*H*$_2$Ctrz), 58.5 (2 × C_q, THQ-4), 56.1 (2 × *C*H$_2$, THQ-3), 50.3 (2 × *C*H$_2$, *C*H$_2$Ntrz), 41.8 (2 × C_q, *C*(CH$_3$)$_2$), 29.6 (2 × CH_3), 29.3 (2 × CH_3), 28.7 (2 × CH_3), 25.5 (2 × CH_3, acetyl). [α]$_D^{20}$: +301° (c=0.46, $CHCl_3$). ESI-MS *m/z*: obsd 1285.53 $[M + H]^+$. HRMS *m/z* calcd for $C_{78}H_{80}N_{10}O_8$ $+H^+$: 1285.62517, obsd 1285.62573, calcd for $C_{78}H_{80}N_{10}O_8 + 2H^+$: 643.31531, obsd 643.31519.

Dimeric ligand (*R*,*R*)-15d. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.63 (s, 2H, 2 × N*H*CO), 7.98 (d, *J* = 8.4, 4H, 4 × CH Ar), 7.76 (s, 2H, 2 × CH Trz), 7.74 (dd, *J* = 8.4, 2.4, 2H, 2 × CH Ar, THQ-8), 7.66 (d, *J* = 7.8, 4H, 4 × CH Ar), 7.59 (d, *J* = 7.2, 6H, 6 × CH Ar), 7.45 (t, *J* = 7.8, 4H, 4 × CH Ar), 7.38 (t, *J* = 7.2, 2H, 2 × CH Ar), 6.96 (d, *J* = 9.0, 4H, 4 × CH Ar), 6.91 (d, *J* = 8.4, 2H, 2 × CH Ar, THQ-7), 6.76 (d, *J* = 9.0, 4H, 4 × CH Ar), 5.06 (s, 4H, 2 × C*H*$_2$, OC*H*$_2$Ctrz), 4.46 (t, *J* = 4.8, 5.4, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 3.78 – 3.73 (m, 4H, 2 × C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.51 (s, 8H, 4 × C*H*$_2$, OC*H*$_2$C*H*$_2$O), 2.45 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.90 (s, 6H, 2 × CH_3, acetyl), 1.76 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.56 (s, 6H, 2 × CH_3), 1.34 (s, 6H, 2 × CH_3), 1.22 (s, 6H, 2 × CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.2 (2 × *C*=O, acetyl), 165.7 (2 × *C*=O, amide), 156.5 (2 × *C*$_q$(OCH$_2$Trz)), 144.6, 143.8, 141.7, 139.7, 137.5, 135.7, 135.7, 133.4 (16 × C_q Ar), 128.9 (4 × CH Ar), 128.1 (2 × CH Ar), 128.0 (4 × CH Ar), 127.7, 127.3, 127.1 (12 × CH Ar), 126.6 (2 × CH Ar, THQ-7), 124.0 (2 × CH Trz), 118.7 (2 × CH Ar, THQ-8), 117.6 (2 × CH Ar, THQ-10), 114.1 (4 × CH Ar), 70.4, 70.3 (4 × *C*H$_2$, OC*H*$_2$*C*H$_2$O), 69.2 (2 × *C*H$_2$, NtrzCH$_2$*C*H$_2$O), 61.8 (2 × *C*H$_2$, OC*H*$_2$Ctrz), 58.5 (2 × C_q, THQ-4), 56.1 (2 × *C*H$_2$, THQ-3), 50.2 (2 × *C*H$_2$, *C*H$_2$Ntrz), 41.8 (2 × C_q, *C*(CH$_3$)$_2$), 29.5 (2 × CH_3), 29.3 (2 × CH_3), 28.7 (2 × CH_3), 25.5 (2 × CH_3, acetyl). [α]$_D^{20}$: +318° (c=0.43, $CHCl_3$). ESI-MS *m/z*: obsd 1329.53 $[M + H]^+$. HRMS *m/z* calcd for $C_{80}H_{84}N_{10}O_9$ $+H^+$: 1329.64955, obsd 1329.65149, calcd for $C_{80}H_{84}N_{10}O_9 + 2H^+$: 665.32841, obsd 665.32849.

Dimeric ligand (*R*,*R*)-15e. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.54 (s, 2H, 2 × N*H*CO), 7.98 (d, *J* = 8.4, 4H, 4 × CH Ar), 7.81 (br s, 2H, 2 × CH Trz), 7.74 (dd, *J* = 8.4, 1.8, 2H, 2 × CH Ar, THQ-8), 7.67 (d, *J* = 8.4, 4H, 4 × CH Ar), 7.60 (d, *J* = 7.8, 4H, 4 × CH Ar), 7.59 (s, 2H, 2 × CH Ar, THQ-10), 7.46 (t, *J* = 7.2, 7.8, 4H, 4 × CH Ar), 7.39 (t, *J* = 7.5, 2H, 2 × CH Ar), 6.97 (d, *J* = 8.4, 4H, 4 × CH Ar), 6.92 (d, *J* = 8.4, 2H, 2 × CH Ar, THQ-7), 6.78 (d, *J* = 8.4, 4H, 4 × CH Ar), 5.07 (s, 4H, 2 × C*H*$_2$, OC*H*$_2$Ctrz), 4.47 (t, *J* = 4.8, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 3.78 (t, *J* = 4.8, 5.4, 4H, 2 × C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.58-3.48 (m, 12H, 6 × C*H*$_2$, OC*H*$_2$C*H*$_2$O), 2.46 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.91 (s, 6H, 2 × CH_3, acetyl), 1.76 (d, *J* = 14.4, 2H, 2 × C*H*$_2$, THQ), 1.57 (s, 6H, 2 × CH_3), 1.34 (s, 6H, 2 × CH_3), 1.22 (s, 6H, 2 × CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (2 × *C*=O, acetyl), 165.6 (2 × *C*=O, amide), 156.6 (2 × *C*$_q$(OCH$_2$Trz)), 144.6, 141.7, 139.8, 137.5, 135.7, 135.6, 133.4 (14 × C_q Ar), 128.9 (4 × CH Ar), 128.1 (2 × CH Ar), 128.0 (4 × CH Ar), 127.7, 127.3, 127.1 (12 × CH Ar), 126.6 (2 × CH Ar, THQ-7), 118.7 (2 × CH Ar, THQ-8), 117.6 (2 × CH Ar, THQ-10), 114.1 (4 × CH Ar), 70.4, 70.3 (6 × *C*H$_2$, OC*H*$_2$*C*H$_2$O), 69.2 (2 × *C*H$_2$, NtrzCH$_2$*C*H$_2$O), 61.8 (2 × *C*H$_2$, OC*H*$_2$Ctrz), 58.5 (2 × C_q, THQ-4), 56.1 (2 × *C*H$_2$, THQ-3), 50.3 (2 × *C*H$_2$, *C*H$_2$Ntrz), 41.8 (2 × C_q, *C*(CH$_3$)$_2$), 29.6 (2 × CH_3), 29.3 (2 × CH_3), 28.7 (2 × CH_3), 25.6 (2 × CH_3, acetyl). [α]$_D^{20}$: +309° (c=0.39, $CHCl_3$). ESI-MS *m/z*: obsd 1373.60 $[M + H]^+$. HRMS *m/z* calcd $C_{82}H_{88}N_{10}O_{10}$ + H^+: 1373.67577, obsd 1373.67651, calcd for $C_{82}H_{88}N_{10}O_{10} + 2H^+$:

687.34152, obsd 687.34143.

Dimeric ligand (*S*,*S*)-15a. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.60 (s, 2H, 2 × N*H*CO), 7.96 (d, *J* = 7.8, 4H, 4 × CH Ar), 7.69 – 7.65 (m, 8H, 8 × CH Ar), 7.60 (d, *J* = 7.2, 4H, 4 × CH Ar), 7.46 (t, *J* = 7.5, 4H, 4 × CH Ar), 7.39 (t, *J* = 7.5, 2H, 2 × CH Ar), 6.96 (d, *J* = 8.4, 4H, 4 × CH Ar), 6.89 (d, *J* = 8.4, 2H, 2 × CH Ar, THQ-7), 6.75 (d, *J* = 8.4, 4H, 4 × CH Ar), 5.04 (q, *J* = 12, 4H, 2 × C*H*$_2$, OC*H*$_2$Ctrz), 4.87 (s, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 2.45 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.87 (s, 6H, 2 × CH_3, acetyl), 1.75 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.56 (s, 6H, 2 × CH_3), 1.35 (s, 6H, 2 × CH_3), 1.21 (s, 6H, 2 × CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (2 × *C*=O, acetyl), 165.7 (2 × *C*=O, amide), 156.3 (2 × *C*$_q$(OCH$_2$Trz)), 144.5, 141.7, 139.7, 137.5, 135.7, 135.62, 133.3 (14 × C_q Ar), 128.9 (4 × CH Ar), 128.1 (2 × CH Ar), 128.0 (4 × CH Ar), 127.7, 127.2, 127.1 (12 × CH Ar), 126.6 (2 × CH Ar, THQ-7), 118.6 (2 × CH Ar, THQ-8), 117.5 (2 × CH Ar, THQ-10), 114.2 (4 × CH Ar), 61.5 (2 × *C*H$_2$, OC*H*$_2$Ctrz), 58.4 (2 × C_q, THQ-4), 56.1 (2 × *C*H$_2$, THQ-3), 49.5 (2 × *C*H$_2$, *C*H$_2$Ntrz), 41.8 (2 × C_q, *C*(CH$_3$)$_2$), 29.4 (2 × CH_3), 29.3 (2 × CH_3), 28.7 (2 × CH_3), 25.4 (2 × CH_3, acetyl). $[\alpha]_D^{20}$: -343° (c=0.49, $CHCl_3$). ESI-MS *m/z*: obsd 1197.53 $[M + H]^+$. HRMS *m/z* calcd $C_{74}H_{72}N_{10}O_6 + H^+$: 1197.57091, obsd 1197.57238, calcd for $C_{74}H_{72}N_{10}O_6 + 2H^+$: 599.28909, obsd 599.28895.

Dimeric ligand (*S*,*S*)-15b. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.71 (s, 2H, 2 × N*H*CO), 7.98 (d, *J* = 8.4, 4H, 4 × CH Ar), 7.73 (dd, *J* = 8.4, 1.8, 2H, 2 × CH Ar, THQ-8), 7.66 (d, *J* = 7.8, 4H, 4 × CH Ar), 7.64 (s, 2H, 2 × CH Ar, THQ-10), 7.60 (d, *J* = 7.8, 4H, 4 × CH Ar), 7.49 (s, 2H, 2 × CH Trz), 7.47 (t, *J* = 7.8, 4H, 4 × CH Ar), 7.40 (t, *J* = 7.2, 2H, 2 × CH Ar), 6.96 (d, *J* = 8.4, 4H, 4 × CH Ar), 6.89 (d, *J* = 8.4, 2H, 2 × CH Ar, THQ-7), 6.77 (d, *J* = 9.0, 4H, 4 × CH Ar), 5.09 (s, 4H, 2 × C*H*$_2$, OC*H*$_2$Ctrz), 4.46 – 4.40 (m, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 3.76 (t, *J* = 4.8, 4H, 2 × C*H*$_2$, C*H*$_2$OC*H*$_2$), 2.45 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.89 (s, 6H, 2 × CH_3, acetyl), 1.75 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.61 (s, 6H, 2 × CH_3), 1.34 (s, 6H, 2 × CH_3), 1.21 (s, 6H, 2 × CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.2 (2 × *C*=O, acetyl), 165.8 (2 × *C*=O, amide), 156.5 (2 × *C*$_q$(OCH$_2$Trz)), 144.6, 144.1, 141.7, 139.8, 137.5, 135.8, 135.7, 133.4 (16 × C_q Ar), 128.9 (4 × CH Ar), 128.1 (2 × CH Ar), 128.0 (4 × CH Ar), 127.8, 127.2, 127.1 (12 × CH Ar), 126.6 (2 × CH Ar, THQ-7), 123.8 (2 × CH Trz), 118.7 (2 × CH Ar, THQ-8), 117.6 (2 × CH Ar, THQ-10), 114.2 (4 × CH Ar), 69.2 (2 × *C*H$_2$, *C*H$_2$O*C*H$_2$), 61.7 (2 × *C*H$_2$, O*C*H$_2$Ctrz), 58.5 (2 × C_q, THQ-4), 56.1 (2 × *C*H$_2$, THQ-3), 50.1 (2 × *C*H$_2$, *C*H$_2$Ntrz), 41.8 (2 × C_q, *C*(CH$_3$)$_2$), 29.6 (2 × CH_3), 29.3 (2 × CH_3), 28.7 (2 × CH_3), 25.5 (2 × CH_3, acetyl). $[\alpha]_D^{20}$: -328° (c=0.44, $CHCl_3$). ESI-MS *m/z*: obsd 1241.53 $[M + H]^+$. HRMS *m/z* calcd $C_{76}H_{76}N_{10}O_7 + H^+$: 1241.59712, obsd 1241.59863, calcd for $C_{76}H_{76}N_{10}O_7 + 2H^+$: 621.30220, obsd 621.30225.

Dimeric ligand (*S*,*S*)-15c. ^{1}H NMR (150 MHz, $CDCl_3$) δ 8.65 (s, 2H, 2 × N*H*CO), 7.97 (d, *J* = 8.4, 4H, 4 × CH Ar), 7.74 (d, *J* = 7.2, 4H, 4 × CH Ar), 7.65 (d, *J* = 8.4, 4H, 4 × CH Ar), 7.59 (d, *J* = 7.8, 6H, 6 × CH Ar), 7.45 (t, *J* = 7.5, 4H, 4 × CH Ar), 7.38 (t, *J* = 7.5, 2H, 2 × CH Ar), 6.96 (d, *J* = 8.4, 4H, 4 × CH Ar), 6.90 (d, *J* = 8.4, 2H, 2 × CH Ar, THQ-7), 6.77 (d, *J* = 6.6, 4H, 4 × CH Ar), 5.08 (s, 4H, 2 × C*H*$_2$, OC*H*$_2$Ctrz), 4.44 (s, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 3.75 (s, 4H, 2 × C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.45 (s, 4H, 2 × C*H*$_2$, OC*H*$_2$C*H*$_2$O), 2.43 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.90 (s, 6H, 2 × CH_3, acetyl), 1.75 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.55 (s, 6H, 2 × CH_3), 1.34 (s, 6H, 2 × CH_3), 1.21 (s, 6H, 2 × CH_3).^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (2 × *C*=O, acetyl), 165.7 (2 × *C*=O, amide), 156.5 (2 × *C*$_q$(OCH$_2$Trz)), 144.5, 141.7, 139.7, 137.5, 135.7, 135.6, 133.4 (14 × C_q Ar), 128.9 (4 × CH Ar), 128.1 (2 × CH Ar), 128.0 (4 × CH Ar), 127.7, 127.2, 127.1 (12 × CH Ar), 126.5 (2 × CH Ar, THQ-7), 118.7 (2 × CH Ar, THQ-8), 117.6 (2 × CH Ar, THQ-10), 114.1 (4 × CH Ar), 70.3 (2 × *C*H$_2$, OCH$_2$*C*H$_2$O), 69.2 (2 × *C*H$_2$, NtrzCH$_2$*C*H$_2$O), 61.8 (2 × *C*H$_2$, O*C*H$_2$Ctrz), 58.4 (2 × C_q, THQ-4), 56.1 (2 × *C*H$_2$, THQ-3), 50.4 (2 × *C*H$_2$, *C*H$_2$Ntrz), 41.8 (2 × C_q, *C*(CH$_3$)$_2$), 29.4 (2 × CH_3), 29.3 (2 × CH_3), 28.7 (2 × CH_3), 25.5 (2 × CH_3, acetyl). $[\alpha]_D^{20}$: -331° (c=0.60, $CHCl_3$). ESI-MS *m/z*: obsd 1285.53 $[M + H]^+$. HRMS *m/z* calcd for $C_{78}H_{80}N_{10}O_8 + H^+$: 1285.62334, obsd 1285.62573, calcd for $C_{78}H_{80}N_{10}O_8 + 2H^+$: 643.31531, obsd 643.31537.

Dimeric ligand (*S*,*S*)-15d. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.83 (s, 2H, 2 × N*H*CO), 8.00 (d, *J* = 8.4, 4H, 4 × CH Ar), 7.79 (s, 2H, 2 × CH Trz), 7.77 (dd, *J* = 8.4, 2.4, 2H, 2 × CH Ar, THQ-8), 7.66 (d, *J* = 8.4, 4H, 4 × CH Ar), 7.65 (s, 2H, 2 × CH Ar, THQ-10), 7.60 (d, *J* = 7.2, 4H, 4 × CH Ar), 7.46 (t, *J* = 7.2, 7.8, 4H, 4 × CH Ar), 7.39 (t, *J* = 7.2, 2H, 2 × CH Ar), 6.98 (d, *J* = 8.4, 4H, 4 × CH Ar), 6.91 (d, *J* = 8.4, 2H, 2 × CH Ar, THQ-7), 6.78 (d, *J* = 9.0, 4H, 4 × CH Ar), 5.07 (s, 4H, 2 × C*H*$_2$, OC*H*$_2$Ctrz), 4.47 (t, *J* = 4.8, 5.4, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 3.77 (t, *J* = 6.0, 7.8, 4H, 2 × C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.52 (s, 8H, 4 × C*H*$_2$, OC*H*$_2$C*H*$_2$O), 2.46 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.92 (s, 6H, 2 × CH_3, acetyl), 1.77 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.57 (s, 6H, 2 × CH_3), 1.35 (s, 6H, 2 × CH_3), 1.23 (s, 6H, 2 × CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.0 (2 × *C*=O, acetyl), 165.7 (2 × *C*=O, amide), 156.5 (2 × *C*$_q$(OCH$_2$Trz)), 144.4, 141.5, 139.7, 137.4, 135.7, 135.5, 133.4 (14 × C$_q$ Ar), 128.8 (4 × CH Ar), 128.0 (2 × CH Ar), 127.9 (4 × CH Ar), 127.7, 127.1, 127.0 (12 × CH Ar), 126.4 (2 × CH Ar, THQ-7), 124.0 (2 × CH Trz), 118.6 (2 × CH Ar, THQ-8), 117.5 (2 × CH Ar, THQ-10), 114.0 (4 × CH Ar), 70.3, 70.2 (4 × *C*H$_2$, OC*H*$_2$*C*H$_2$O), 69.1 (2 × *C*H$_2$, NtrzCH$_2$*C*H$_2$O), 61.7 (2 × *C*H$_2$, OC*H*$_2$Ctrz), 58.4 (2 × C$_q$, THQ-4), 56.0 (2 × *C*H$_2$, THQ-3), 50.1 (2 × *C*H$_2$, *C*H$_2$Ntrz), 41.7 (2 × C$_q$, *C*(CH$_3$)$_2$), 29.5 (2 × CH_3), 29.2 (2 × CH_3), 28.6 (2 × CH_3), 25.3 (2 × CH_3, acetyl). [α]$_D^{20}$: -329° (c=0.65, $CHCl_3$). ESI-MS *m/z*: obsd 1329.53 $[M + H]^+$. HRMS *m/z* calcd for $C_{80}H_{84}N_{10}O_9$ $+H^+$: 1329.64955, obsd 1329.65076, calcd for $C_{80}H_{84}N_{10}O_9 + 2H^+$: 665.32841, obsd 665.32643.

Dimeric ligand (*S*,*S*)-15e. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.67 (s, 2H, 2 × N*H*CO), 7.99 (d, *J* = 8.4, 4H, 4 × CH Ar), 7.82 (br s, 2H, 2 × CH Trz), 7.76 (dd, *J* = 8.4, 2.4, 1.8, 2H, 2 × CH Ar, THQ-8), 7.67 (d, *J* = 7.8, 4H, 4 × CH Ar), 7.60 (d, *J* = 7.2, 6H, 6 × CH Ar), 7.46 (t, *J* = 7.8, 4H, 4 × CH Ar), 7.39 (t, *J* = 7.2, 2H, 2 × CH Ar), 6.98 (d, *J* = 8.4, 4H, 4 × CH Ar), 6.92 (d, *J* = 8.4, 2H, 2 × CH Ar, THQ-7), 6.79 (d, *J* = 8.4, 4H, 4 × CH Ar), 5.07 (s, 4H, 2 × C*H*$_2$, OC*H*$_2$Ctrz), 4.48 (t, *J* = 4.8, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 3.79 (t, *J* = 4.8, 4.8, 4H, 2 × C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.55, 3.53 (2 × s, 12H, 6 × C*H*$_2$, OC*H*$_2$C*H*$_2$O), 2.46 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.92 (s, 6H, 2 × CH_3, acetyl), 1.76 (d, *J* = 14.4, 2H, 2 × C*H*$_2$, THQ), 1.57 (s, 6H, 2 × CH_3), 1.34 (s, 6H, 2 × CH_3), 1.22 (s, 6H, 2 × CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (2 × *C*=O, acetyl), 165.7 (2 × *C*=O, amide), 156.6 (2 × *C*$_q$(OCH$_2$Trz)), 144.5, 141.7, 139.8, 137.5, 135.7, 135.7, 133.4 (14 × C$_q$ Ar), 128.9 (4 × CH Ar), 128.1 (2 × CH Ar), 128.0 (4 × CH Ar), 127.8, 127.2, 127.1 (12 × CH Ar), 126.6 (2 × CH Ar, THQ-7), 118.7 (2 × CH Ar, THQ-8), 117.6 (2 × CH Ar, THQ-10), 114.1 (4 × CH Ar), 70.5, 70.4, 70.3 (6 × *C*H$_2$, OC*H*$_2$*C*H$_2$O), 69.2 (2 × *C*H$_2$, NtrzCH$_2$*C*H$_2$O), 61.8 (2 × *C*H$_2$, OC*H*$_2$Ctrz), 58.5 (2 × C$_q$, THQ-4), 56.1 (2 × *C*H$_2$, THQ-3), 50.3 (2 × *C*H$_2$, *C*H$_2$Ntrz), 41.8 (2 × C$_q$, *C*(CH$_3$)$_2$), 29.6 (2 × CH_3), 29.3 (2 × CH_3), 28.7 (2 × CH_3), 25.5 (2 × CH_3, acetyl). [α]$_D^{20}$: -328° (c=0.46, $CHCl_3$). ESI-MS *m/z*: obsd 1373.60 $[M + H]^+$. HRMS *m/z* calcd $C_{82}H_{88}N_{10}O_{10}$ + H^+: 1373.67577, obsd 1373.67761, calcd for $C_{82}H_{88}N_{10}O_{10}$ + $2H^+$: 687.34152, obsd 687.34131.

General procedure for the preparation of monomeric ligands (R,S)-15a-e.

Monomeric compounds **(*R*)-14a**, **(*R*)-14b**, **(*R*)-14c**, **(*R*)-14d** or **(*R*)-14e** (12.5 μmol) and **(*S*)-2** (8.13 mg, 15.0 μmol) was dissolved in 10 mL DCM. The mixture was degassed under argon in an ultrasonic bath, until the volume was approximately 1.5 mL. Water and solutions of $CuSO_4$ and sodium ascorbate were degassed separately. Water (1.0 mL) and aqueous solutions of sodium ascorbate (50 μL, 1 eq) and $CuSO_4$ (50 μL, 20 mol%) were added. After stirring for 18 h, TLC and LC-MS showed all reactions to be completed. Reaction mixtures were concentrated and coevaporated with toluene (3×). The residue was dissolved in DCM and the product was isolated using silica column chromatography (0 to 3,5% MeOH in DCM). Pure products were obtained as white powdery crystals in 88-100% yield. *R*f = 0.45 (10% MeOH in DCM).

Dimeric ligand (*R*,*S*)-15a. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.49 (s, 2H, 2 × N*H*CO), 7.97 (d, *J* = 8.4, 4H, 4 × CH Ar), 7.69-7.66 (m, 6H, 6 × CH Ar), 7.60 (d, *J* = 7.8, 6H, 6 × CH Ar), 7.46 (t, *J* = 7.5, 4H, 4 × CH Ar), 7.39 (t, *J* = 7.2, 2H, 2 × CH Ar), 7.31 (s, 2H, 2 × CH Trz), 6.96 (d, *J* = 8.4, 4H, 4 × CH Ar), 6.90 (d, *J* = 8.4, 2H, 2 × CH Ar, THQ-7), 6.74 (d, *J* = 8.4, 4H, 4 × CH Ar), 5.05 (s, 4H, 2 × C*H*$_2$, OC*H*$_2$Ctrz), 4.86 (s, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 2.44

(d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.88 (s, 6H, 2 × CH_3, acetyl), 1.74 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.56 (s, 6H, 2 × CH_3), 1.32 (s, 6H, 2 × CH_3), 1.20 (s, 6H, 2 × CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (2 × *C*=O, acetyl), 165.7 (2 × *C*=O, amide), 156.3 (2 × *C*$_q$(OCH$_2$Trz)), 144.6, 144.4, 141.8, 139.8, 137.6, 135.7, 135.7, 133.3 (16 × C_q Ar), 129.0 (4 × CH Ar), 128.1 (2 × CH Ar), 128.1 (4 × CH Ar), 127.7, 127.3, 127.2 (12 × CH Ar), 126.7 (2 × CH Ar, THQ-7), 123.9 (2 × CH Trz), 118.8 (2 × CH Ar, THQ-8), 117.6 (2 × CH Ar, THQ-10), 114.3 (4 × CH Ar), 61.7 (2 x *C*H$_2$, OC*H*$_2$Ctrz), 58.5 (2 × C_q, THQ-4), 56.1 (2 × *C*H$_2$, THQ-3), 49.5 (2 × *C*H$_2$, *C*H$_2$Ntrz), 41.9 (2 × C_q, *C*(CH$_3$)$_2$), 29.4 (2 × CH_3), 29. (2 × CH_3), 28.8 (2 × CH_3), 25.5 (2 × CH_3, acetyl). [α]$_D^{20}$: -2° (c=0.35, $CHCl_3$). ESI-MS *m/z*: obsd 1197.8 [M + H]$^+$. HRMS *m/z* calcd $C_{74}H_{72}N_{10}O_6$ + H$^+$: 1197.57091, obsd 1197.57227, calcd for $C_{74}H_{72}N_{10}O_6$ + 2H$^+$: 599.28909, obsd 599.28906.

Dimeric ligand (*R*,*S*)-15b. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.60 (s, 2H, 2 × N*H*CO), 8.01 (d, *J* = 7.8, 4H, 4 × CH Ar), 7.74 (dd, *J* = 8.4, 1.8, 2H, 2 × CH Ar, THQ-8), 7.68 (d, *J* = 7.8, 4H, 4 × CH Ar), 7.61 (d, *J* = 7.8, 4H, 4 × CH Ar), 7.56 (s, 2H, 2 × CH Ar, THQ-10), 7.53 (br s, 2H, 2 × CH Trz), 7.47 (t, *J* = 7.2, 7.8, 4H, 4 × CH Ar), 7.40 (t, *J* = 7.5, 2H, 2 × CH Ar), 6.96 (d, *J* = 8.4, 4H, 4 × CH Ar), 6.91 (d, *J* = 8.4, 2H, 2 × CH Ar, THQ-7), 6.79 (d, *J* = 7.8, 4H, 4 × CH Ar), 5.12 (s, 4H, 2 × C*H*$_2$, OC*H*$_2$Ctrz), 4.42 (s, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 3.75 (t, *J* = 6.0, 4H, 2 × C*H*$_2$, C*H*$_2$OC*H*$_2$), 2.45 (d, *J* = 14.4, 2H, 2 × C*H*$_2$, THQ), 1.88 (s, 6H, 2 × CH_3, acetyl), 1.75 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.60 (s, 6H, 2 × CH_3), 1.33 (s, 6H, 2 × CH_3), 1.21 (s, 6H, 2 × CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (2 × *C*=O, acetyl), 165.7 (2 × *C*=O, amide), 156.4 (2 × *C*$_q$(OCH$_2$Trz)), 144.6, 141.6, 139.7, 137.5, 135.7, 135.6, 133.3 (14 × C_q Ar), 128.9 (4 × CH Ar), 128.1 (2 × CH Ar), 128.0 (4 × CH Ar), 127.7, 127.3, 127.1 (12 × CH Ar), 126.6 (2 × CH Ar, THQ-7), 118.8 (2 × CH Ar, THQ-8), 117.6 (2 × CH Ar, THQ-10), 114.2 (4 × CH Ar), 69.2 (2 × *C*H$_2$, *C*H$_2$O*C*H$_2$), 61.7 (2 × *C*H$_2$, OCH$_2$Ctrz), 58.4 (2 × C_q, THQ-4), 56.1 (2 × *C*H$_2$, THQ-3), 50.1 (2 × *C*H$_2$, *C*H$_2$Ntrz), 41.8 (2 × C_q, *C*(CH$_3$)$_2$), 29.5 (2 × CH_3), 29.3 (2 × CH_3), 28.7 (2 × CH_3), 25.5 (2 × CH_3, acetyl). [α]$_D^{20}$: -9° (c=0.37, $CHCl_3$). ESI-MS *m/z*: obsd 1241.7 [M + H]$^+$. HRMS *m/z* calcd $C_{76}H_{76}N_{10}O_7$ + H$^+$: 1241.59712, obsd 1241.59863, calcd for $C_{76}H_{76}N_{10}O_7$ + 2H$^+$: 621.30220, obsd 621.30200.

Dimeric ligand (*R*,*S*)-15c. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.54 (s, 2H, 2 × N*H*CO), 7.99 (d, *J* = 8.4, 4H, 4 × CH Ar, 7.76 (d, *J* = 7.8, 2H, 2 × CH Ar, THQ-8), 7.67 (d, *J* = 7.8, 4H, 4 × CH Ar), 7.60 (d, *J* = 7.2, 4H, 4 × CH Ar), 7.52 (s, 2H, 2 × CH Ar, THQ-10), 7.46 (t, *J* = 7.5, 4H, 4 × CH Ar), 7.39 (t, *J* = 7.2, 2H, 2 × CH Ar), 6.96 (d, *J* = 7.8, 4H, 4 × CH Ar), 6.92 (d, *J* = 8.4, 2H, 2 × CH Ar, THQ-7), 6.78 (s, 4H, 4 × CH Ar), 5.09 (s, 4H, 2 × C*H*$_2$, OC*H*$_2$Ctrz), 4.44 (s, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 3.75 (s, 4H, 2 × C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.45 (s, 4H, 2 × C*H*$_2$, OC*H*$_2$C*H*$_2$O), 2.44 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.91 (s, 6H, 2 × CH_3, acetyl), 1.75 (d, *J* = 13.8, 2H, 2 × C*H*$_2$, THQ), 1.57 (s, 6H, 2 × CH_3), 1.34 (s, 6H, 2 × CH_3), 1.22 (s, 6H, 2 × CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (2 × *C*=O, acetyl), 165.6 (2 × *C*=O, amide), 156.5 (2 × *C*$_q$(OCH$_2$Trz)), 144.6, 141.7, 139.8, 137.5, 135.7, 135.6, 133.4 (14 × C_q Ar), 128.9 (4 × CH Ar), 128.1 (2 × CH Ar), 128.0 (4 × CH Ar), 127.7, 127.3, 127.1 (12 × CH Ar), 126.6 (2 × CH Ar, THQ-7), 118.7 (2 × CH Ar, THQ-8), 117.6 (2 × CH Ar, THQ-10), 114.1 (4 × CH Ar), 70.3 (2 × *C*H$_2$, OC*H*$_2$*C*H$_2$O), 69.2 (2 × *C*H$_2$, NtrzCH$_2$*C*H$_2$O), 61.8 (2 × OCH$_2$Ctrz), 58.4 (2 × C_q, THQ-4), 56.1 (2 × *C*H$_2$, THQ-3), 50.8 (2 × *C*H$_2$, *C*H$_2$Ntrz), 41.8 (2 × C_q, *C*(CH$_3$)$_2$), 29.3 (4 × CH_3), 28.7 (2 × CH_3), 25.5 (2 × CH_3, acetyl). [α]$_D^{20}$: -10° (c=0.33, $CHCl_3$). ESI-MS *m/z*: obsd 1285.7 [M + H]$^+$. HRMS *m/z* calcd for $C_{78}H_{80}N_{10}O_8$ +H$^+$: 1285.62334, obsd 1285.62476, calcd for $C_{78}H_{80}N_{10}O_8$ + 2H$^+$: 643.31531, obsd 643.31531.

Dimeric ligand (*R*,*S*)-15d. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.57 (s, 2H, 2 × N*H*CO), 7.99 (d, *J* = 8.4, 4H, 4 × CH Ar), 7.83 (br s, 2H, 2 × CH Trz), 7.75 (d, *J* = 8.4, 2H, 2 × CH Ar, THQ-8), 7.67 (d, *J* = 7.2, 4H, 4 × CH Ar), 7.60 (d, *J* = 7.8, 4H, 4 CH Ar), 7.57 (s, 2H, 2 × CH Ar, THQ-10), 7.46 (t, *J* = 7.8, 4H, 4 × CH Ar), 7.39 (t, *J* = 7.2, 2H, 2 × CH Ar), 6.97 (d, *J* = 7.8, 4H, 4 × CH Ar), 6.91 (d, *J* = 8.4, 2H, 2 × CH Ar, THQ-7), 6.78 (d, *J* = 7.2, 4H, 4 × CH Ar), 5.07 (s, 4H, 2 × C*H*$_2$, OC*H*$_2$Ctrz), 4.46 (s, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 3.76 (s, 4H, 2 × C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.50 (s,

8H, 4 × CH_2, OCH_2CH_2O), 2.45 (d, *J* = 13.8, 2H, 2 × CH_2, THQ), 1.91 (s, 6H, 2 × CH_3, acetyl), 1.76 (d, *J* = 13.8, 2H, 2 × CH_2, THQ), 1.55 (s, 6H, 2 × CH_3), 1.33 (s, 6H, 2 × CH_3), 1.22 (s, 6H, 2 × CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (2 × *C*=O, acetyl), 165.6 (2 × *C*=O, amide), 156.5 (2 × C_q(OCH_2Trz)), 144.6, 141.6, 139.7, 137.5, 135.7, 135.6, 133.3 (14 × C_q Ar), 128.9 (4 × CH Ar), 128.1 (2 × CH Ar), 128.0 (4 × CH Ar), 127.7, 127.2, 127.1 (12 × CH Ar), 126.5 (2 × CH Ar, THQ-7), 118.7 (2 × CH Ar, THQ-8), 117.6 (2 × CH Ar, THQ-10), 114.0 (4 × CH Ar), 70.4, 70.3 (4 × *C*H_2, OCH_2*C*H_2O), 69.1 (2 × *C*H_2, NtrzCH_2*C*H_2O), 61.8 (2 × *C*H_2, O*C*H_2Ctrz), 58.4 (2 × C_q, THQ-4), 56.0 (2 × *C*H_2, THQ3), 50.3 (2 x *C*H_2, *C*H_2Ntrz), 41.8 (2 × C_q, *C*$(CH_3)_2$), 29.5 (2 × CH_3), 29.3 (2 × CH_3), 28.7 (2 × CH_3), 25.4 (2 × CH_3, acetyl). $[\alpha]_D^{20}$: 0° (c=0.34, $CHCl_3$). ESI-MS *m/z*: obsd 665.5 $[M + 2H]^{2+}$. HRMS *m/z* calcd for $C_{80}H_{84}N_{10}O_9$ $+H^+$: 1329.64955, obsd 1329.65173, calcd for $C_{80}H_{84}N_{10}O_9 + 2H^+$: 665.32841, obsd 665.32843.

Dimeric ligand (*R*,*S*)-15e. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.54 (s, 2H, 2 × N*H*CO), 7.99 (d, *J* = 7.8, 4H, 4 × CH Ar), 7.83 (br s, 2H, 2 × CH Trz), 7.75 (d, *J* = 6.6, 2H, 2 × CH Ar, THQ-8), 7.68 (d, *J* = 8.4, 4H, 4 × CH Ar), 7.60 (d, *J* = 7.8, 4H, 4 × CH Ar), 7.57 (s, 2H, CH Ar, THQ-10), 7.46 (t, *J* = 7.2, 7.8, 4H, 4 × CH Ar), 7.39 (t, *J* = 7.5, 2H, 2 × CH Ar), 6.97 (d, *J* = 8.4, 4H, 4 × CH Ar), 6.92 (d, *J* = 8.4, 2H, 2 × CH Ar, THQ-7), 6.79 (d, *J* = 7.8, 4H, 4 × CH Ar), 5.08 (s, 4H, 2 × CH_2, O*C*H_2Ctrz), 4.47 (s, 4H, 2 × CH_2, CH_2Ntrz), 3.78 (t, *J* = 4.2, 4.8, 4H, 2 × CH_2, NtrzCH_2CH_2O), 3.54, 3.52 (2 × s, 12H, 6 × CH_2, OCH_2CH_2O), 2.46 (d, *J* = 14.4, 2H, 2 × CH_2, THQ), 1.91 (s, 6H, 2 × CH_3, acetyl), 1.76 (d, *J* = 14.4, 2H, 2 × CH_2, THQ), 1.56 (s, 6H, 2 × CH_3), 1.33 (s, 6H, 2 × CH_3), 1.22 (s, 6H, 2 × CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1 (2 × *C*=O, acetyl), 165.6 (2 × *C*=O, amide), 156.6 (2 × C_q(OCH_2Trz)), 144.6, 141.7, 139.8, 137.5, 135.7, 135.6, 133.4 (14 × C_q Ar), 128.9 (4 × CH Ar), 128.1 (2 × CH Ar), 128.0 (4 × CH Ar), 127.7, 127.3, 127.2 (12 × CH Ar), 126.6 (2 × CH Ar, THQ-7), 118.7 (2 × CH Ar, THQ-8), 117.6 (2 × CH Ar, THQ-10), 114.1 (4 × CH Ar), 70.4, 70.3 (6 × *C*H_2, O*C*H_2*C*H_2O), 69.2 (2 × *C*H_2, NtrzCH_2*C*H_2O), 61.8 (2 x *C*H_2, O*C*H_2Ctrz), 58.5 (2 × C_q, THQ-4), 56.1 (2 × *C*H_2, THQ-3), 50.3 (2 x *C*H_2, *C*H_2Ntrz), 41.8 (2 × C_q, *C*$(CH_3)_2$), 29.6 (2 × CH_3), 29.3 (2 × CH_3), 28.7 (2 × CH_3), 25.5 (2 × CH_3, acetyl). $[\alpha]_D^{20}$: 0° (c=0.34, $CHCl_3$). ESI-MS *m/z*: obsd 1373.8 $[M + H]^+$. HRMS *m/z* calcd for $C_{82}H_{88}N_{10}O_{10}$ $+H^+$: 1373.67577, obsd 1373.67627, calcd for $C_{82}H_{88}N_{10}O_{10} + 2H^+$: 687.34152, obsd 687.34161.

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Chapter 9

Synthesis and pharmacological evaluation of heterodimeric FSHR and LHR ligands

Introduction

In recent years, several low molecular weight modulators for the FSHR[1-5] and LHR[6-8] have been reported that interact with the transmembrane (TM) region of the receptors. Compound **1** is a potent FSHR antagonist based on a tetrahydroquinoline (THQ) scaffold of which only the (*R*)-enantiomer is active (Figure 1).[1] Compound **2** is a potent LHR agonist that also shows agonistic activity on the FSHR (for clarity here called LH/FSHR agonist **2**)[6].

Dimeric ligands derived from both these modulators were synthesized and tested on the FSHR- and LHR-modulating properties. Dimeric ligands derived from LH/FSHR agonist **2** showed an interesting increase in selectivity for the LHR compared to their monomeric counterparts, as described in Chapter 5. The observed selectivity primarily originated from a significant decrease in efficacy on the FSHR. Dimeric ligands based on FSHR antagonist **(*R*)-1** proved to have increasing potency with increasing spacer length when compared to the dimeric ligands in which one of the recognition heads was replaced by the inactive enantiomer **(*S*)-1** (described in Chapter 8). Both these results indicate that the second pharmacophore played a significant role in the

interaction of the dimeric ligands with the receptors. The exact mode of action of the dimeric ligands proved to be difficult to establish with the compounds presented in Chapters 5 and 8. Here the binding process is further elucidated by the preparation of heterodimeric constructs combining an FSHR antagonist and a LH/FSHR agonist in one molecule, connected by a spacer that is based on a flexible ethylene glycol moiety (Figure 1).

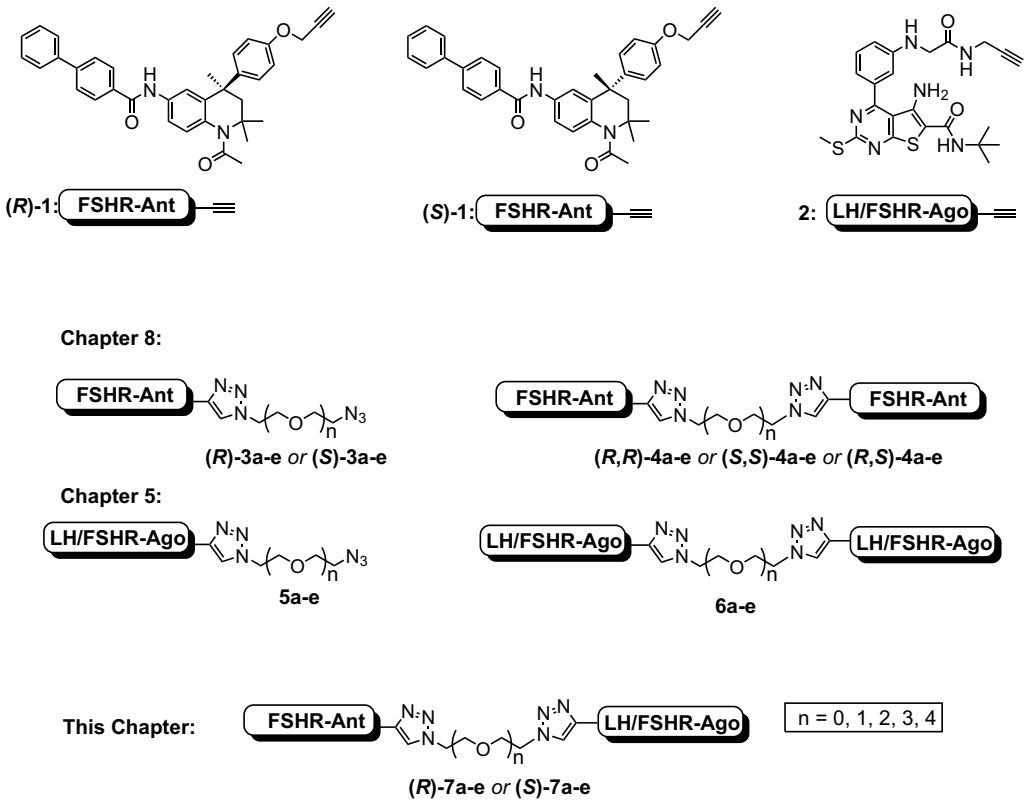
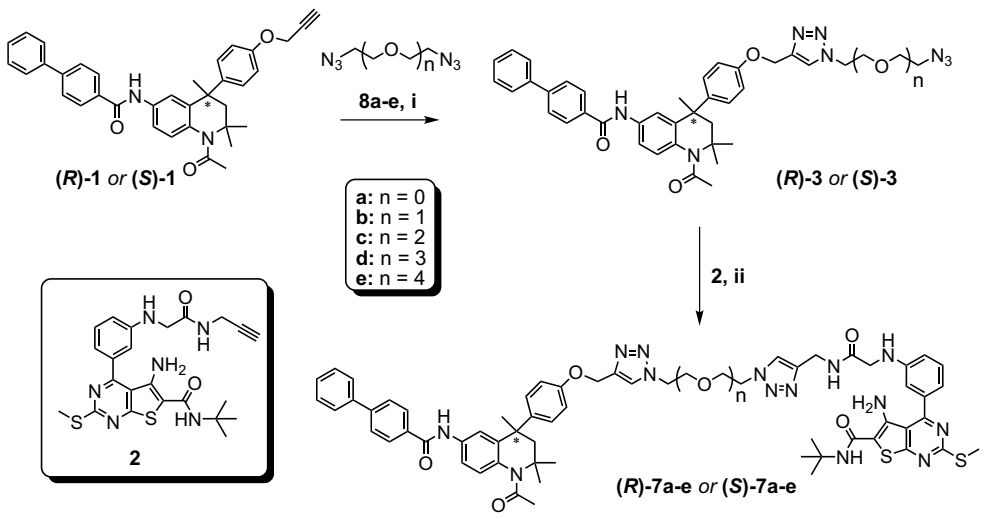


Figure 1. General overview of the compounds described in this Chapter.

Results and discussion

The heterodimeric ligands were prepared from previously described building blocks. Thus, the acetylene functionalized pharmacophores **(*R*)-1** and **(*S*)-1** were reacted with 5 equivalents of bis-azide spacers **8a-e** with copper sulfate and sodium ascorbate in a biphasic water/DCM mixture[9] to give the compounds **(*R*)-3** and **(*S*)-3**. The free azide functions on the spacer termini in **(*R*)-3** and **(*S*)-3** can be used in a second [2+3]-Huisgen cycloaddition reaction with the acetylene functionalized LH/FSHR agonist **2** (Scheme 1). In total, 5 hetero-dimeric ligands that differ in spacer length were prepared that were derived from **(*R*)-1** and **2** (that is, compounds **(*R*)-7**) and 5 reference compounds that were derived from **(*S*)-1** and **2** (that is, compounds **(*S*)-7**).



Scheme 1. Synthesis of heterodimeric FSH-ligands. *Reagents and conditions*: *i*. 5 eq. bis-azide spacer **8a-e**, $CuSO_4$ (0.2 eq) sodium ascorbate (1 eq), DCM/H_2O, 33-56%; *ii*. Acetylene **2**, $CuSO_4$ (0.2 eq), sodium ascorbate (1 eq), DCM/H_2O, 75-97%.

First, the antagonistic potencies of the newly prepared compounds were determined with CHO-K1 cells expressing the human FSHR. The receptor was stimulated to 80% of its maximal efficacy with recombinant FSH in the absence and presence of the test compounds at increasing concentrations. For comparison, the IC_{50} values for the previously prepared monomeric FSH ligands **(*R*)-3a-e** and dimeric FSH ligands **(*R*,*R*)-4a-e** and **(*R*,*S*)-4a-e** are listed together with the here obtained results from hetero-dimeric ligands **(*R*)-7a-e** (Table 1). The compounds **(*S*)-7a-e** did not show any antagonistic activity on the FSHR like monomeric compound **(*S*)-1** and are therefore not listed.

	Spacer (n)	IC_{50} FSHR (nM)		Spacer (n)	IC_{50} FSHR (nM) (I_{max})
(*R*)-1	-	39	**2**	-	n.a.
Monovalent			***Homo-bivalent (R)-(R)-antagonists***		
(*R*)-3a	n = 0	115	**(*R*,*R*)-4a**	n = 0	>10000 *
(*R*)-3b	n = 1	141	**(*R*,*R*)-4b**	n = 1	6725 *
(*R*)-3c	n = 2	324	**(*R*,*R*)-4c**	n = 2	4712
(*R*)-3d	n = 3	379	**(*R*,*R*)-4d**	n = 3	3712
(*R*)-3e	n = 4	482	**(*R*,*R*)-4e**	n = 4	3107
Hetero-bivalent (R)-(S)-antagonists			***Hetero-bivalent antagonist-agonist***		
(*R*,*S*)-4a	n = 0	2660	**(*R*)-7a**	n = 0	65% at 10 µM
(*R*,*S*)-4b	n = 1	2077	**(*R*)-7b**	n = 1	75% at 10 µM
(*R*,*S*)-4c	n = 2	7832 *	**(*R*)-7c**	n = 2	920 (73%)
(*R*,*S*)-4d	n = 3	9015 *	**(*R*)-7d**	n = 3	1662 (65%)
(*R*,*S*)-4e	n = 4	>10000 *	**(*R*)-7e**	n = 4	1023 (72%)

Table 1. Mean antagonistic potency (IC_{50}) and maximal inhibition (I_{max}) of hetero-dimeric compounds derived from a FSHR antagonist and LH/FSHR agonist **(*R*)-7a-e**. For comparison, the potencies for the previously prepared compounds **(*R*)-3a-e**, **(*R*,*R*)-4a-e** and **(*R*,*S*)-4a-e** are listed. I_{max} is the maximal percentage of inhibition of FSH mediated signaling observed for the compound at 10 µM. IC_{50} values are determined from two independent experiments performed in duplicate. SD of pIC_{50} is generally lower than 0.3. *: estimated IC_{50} due to incomplete curve. n.a.: not active.

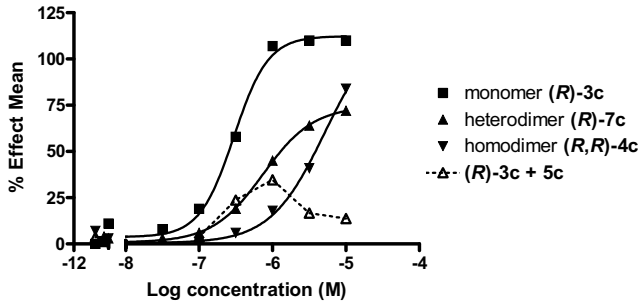


Figure 2. Concentration-effect curves (antagonistic set-up) of selected compounds on CHO-K1 cells expressing FSHR that were stimulated with recFSH. Selected compounds are monomeric FSHR antagonist **(*R*)-3c**, homodimeric FSHR antagonist **(*R*,*R*)-4c** and heterodimeric FSHR antagonist/FSH/LHR agonist **(*R*)-7c**. Mixture of FSHR antagonist **(*R*)-3c** and FSH/LHR agonist **5c** showed dose dependent inhibition up to 1 μM of testcompound. At higher concentration the inhibitory effect was decreased. Concentration-effect curves were determined from two or three independent experiments performed in duplicate

Only the compounds with the longer spacers (that is, **(*R*)-7c-e**) show a maximal antagonistic plateau in the curves, however, these compounds did not antagonize FSH mediated signaling by 100% (see also Figure 2). The compounds with a short spacer (that is, **(*R*)-7a-b**) show less than 75% antagonistic efficacy at 10 μM of test compound. Surprisingly, the potencies for the hetero-dimeric ligands **(*R*)-7c-e** are higher than the homo-dimeric compounds with two FSHR antagonists **(*R*,*R*)4c-e**. The effect of the LH/FSHR agonist and the FSHR antagonist was also investigated when the compounds are not interconnected by a spacer but mixed. The monomeric FSHR antagonist **(*R*)-3c** (IC_{50} = 324 nM) and the monomeric LH/FSHR agonist **5c** (EC_{50} = 363 nM), two compounds with the same spacer length, were combined in equal proportions. Here, at lower concentration of test compound a dose dependent increase of antagonism was detected up to 1 μM after which the antagonism was decreased at higher concentrations (See Figure 2). This typical effect was also observed for other mixtures with different spacer lengths.

Next, the activity of the compounds **(*R*)-7a-e** and **(*S*)-7a-e** on the FSHR and LHR in an agonistic set-up was explored. Here, the test compounds were added to the receptors in the absence of recombinant FSH (or recLH) to investigate the agonistic potencies. For comparison, the EC_{50} values for the earlier prepared monomeric and dimeric LH/FSHR agonists (that is, **5a-e** and **6a-e**), as described in chapter 5, are also listed in Table 2. Monomeric and dimeric compounds derived from FSHR antagonist **(*R*)-1** or **(*S*)-1** were not active in the agonistic assay and are therefore not listed in the table.

All heterodimeric ligands **(*R*)-7a-e** and **(*S*)-7a-e** are potent LHR agonists. The potencies are generally lower when compared to the dimeric ligands with two LHR agonists on either side of the linker (**6a-e**). This confirms the involvement of the second LHR agonist in the obtained potencies of dimeric ligands **6a-e**. The hetero-dimeric compounds **(*R*)-7a-e** with the active (*R*)-FSHR antagonist on one side of the linker show a similar trend as the dimeric LHR agonists **6a-e** in that the potency decreases with increasing spacer length. Within the hetero-dimeric series **(*R*)-7a-e** and **(*S*)-7a-e**, the compounds with short spacers assembled from the (*R*)-enantiomer (**(*R*)-7a-c**)

	Spacer (n)	EC_{50} (nM)			Spacer (n)	EC_{50} (nM)	
		FSHR (E_{max})	LHR			FSHR (E_{max})	LHR
Monovalent LH/FSHR agonist				***Homo-bivalent LH/FSHR agonist***			
2	-	126 (78%)	1				
5a	n = 0	494 (71%)	4	**6a**	n = 0	780 (23%)	16
5b	n = 1	473 (75%)	4	**6b**	n = 1	494 (17%)	19
5c	n = 2	363 (81%)	5	**6c**	n = 2	527 (21%)	23
5d	n = 3	585 (80%)	6	**6d**	n = 3	475 (19%)	25
5e	n = 4	700 (72%)	6	**6e**	n = 4	506 (18%)	28
Hetero-bivalent FSHR antagonist- LH/FSHR agonist							
(*R*)-7a	n = 0	n.a.	10	**(*S*)-7a**	n = 0	43% at 10 μM	24
(*R*)-7b	n = 1	n.a.	27	**(*S*)-7b**	n = 1	44% at 10 μM	80
(*R*)-7c	n = 2	n.a.	43	**(*S*)-7c**	n = 2	18% at 10 μM	75
(*R*)-7d	n = 3	n.a.	61	**(*S*)-7d**	n = 3	31% at 10 μM	57
(*R*)-7e	n = 4	n.a.	55	**(*S*)-7e**	n = 4	39% at 10 μM	39

Table 2. EC_{50} values for heterodimeric compounds **(*R*)-7a-e** and **(*S*)-7a-e**. For comparison, the potencies for the previously prepared monomeric compounds **2** and **5a-e** and dimeric compounds **6a-e** are listed. All compounds are full agonists for the LHR and partial agonists for the FSHR. E_{max} is maximal percentage of effect observed for the compounds when compared to recombinant FSH signaling. EC_{50} values are determined from two independent experiments performed in duplicate. SD of pIC_{50} is generally lower than 0.2. n.a.: not active.

are somewhat more potent than the corresponding compounds containing the (*S*)-enantiomer **(*S*)-7a-c**. The reverse is observed with the longer spacer lengths. Here, the compounds **(*R*)-7e** is less potent than **(*S*)-7e**.

An interesting observation can be made when the activation of the FSHR with the compounds is explored. The hetero-bivalent compounds with on one side the active (*R*)-FSHR antagonist and the other side the LH/FSHR agonist (**(*R*)-7a-e**) did not show any agonistic activity on the FSHR up to 10 μM of test compound. The corresponding compounds with the (*S*)-FSHR antagonist on one side (**(*S*)-7a-e**) were still able to activate the FSHR to some extent at the highest concentration tested (10 μM). The maximal efficacy could not be determined for compounds **(*S*)-7a-e** due to incomplete curves but it could be established that they are less potent on the FSHR than the dimeric ligands with two LH/FSHR agonists on either side of the linker (that is **6a-e**).

It was investigated whether the FSHR agonistic activity of the LH/FSHR agonist can also be reduced when mixing the compounds instead of interconnecting the compounds. A representative example of the results is depicted in Figure 3 in where the monomeric LH/FSHR agonist **5c** was mixed with the active FSHR antagonist **(*R*)-3c**. While the potency of this mixture on the LHR remains in the same order of magnitude as in the absence of (*R*)-antagonist, the potency on the FSHR is reduced significantly ($P<0.05$). This is in accordance with direct displacement of the agonist **5c** by the antagonist **(*R*)-3c**. Thus, when the LH/FSHR agonist is interconnected to the active (*R*)-FSHR antagonist, agonistic activity for the FSH receptor is completely reduced whereas mixing the compounds still results in some FSHR agonistic activity. This demonstrates the additional effect of the recognition units in the dimeric ligand **(*R*)-7c** to interact with the FSHR.

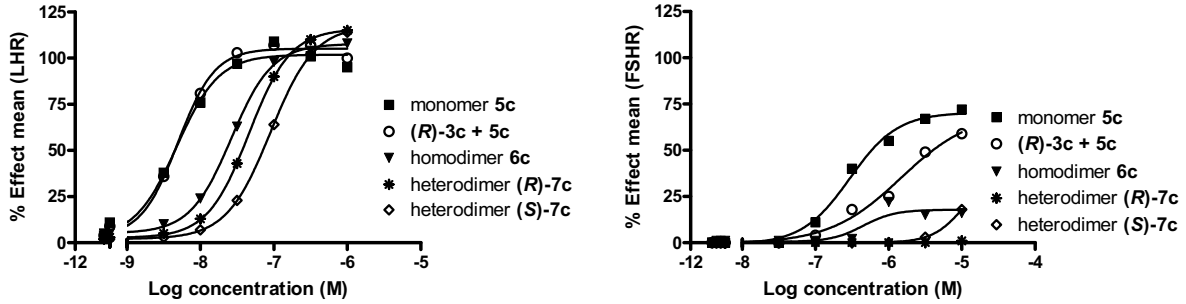


Figure 3. Concentration effect curves (agonistic set-up)of selected compounds on CHO cells expressing either the LHR (left) or the FSHR (right). Selected compounds are monomeric LH/FSHR agonist **5c**, homodimeric LH/FSHR agonist **6c** and heterodimeric FSHR antagonist-FSH/LHR agonists **(*R*)-7c** and **(*S*)-7c**. Mixture of FSHR antagonist **3c** and FSH/LHR agonist **5c** are also depicted. Concentration-effect curves were determined from two or three independent experiments performed in duplicate

Conclusion

In summary, interconnecting an FSHR antagonist to a LH/FSHR agonist provided compounds with unique pharmacological properties that could not be reproduced by solely mixing the independent recognition units. For example, no FSHR agonistic activities were observed for compounds **(*R*)-7a-e**, while the compounds are active agonists on the LHR. Several conclusions may be drawn from the results above. 1) The LHR tolerates the large inactive (*S*)-THQ or active (*R*)-THQ that is connected to lead structure **2** with only a moderate loss in agonistic potency on the receptor. In addition, no discrepancy in activity of the compounds is observed on the LHR if the monomeric units from (*R*)-THQ and **2** are mixed. This suggests that the (*R*)-THQ does not influence the bioactivity of the monomeric ligands derived from **2** on the LHR. 2) The agonistic potency on the FSHR is significantly reduced by introducing the same inactive (*S*)-THQ on **2**. However at the highest concentration of test compound, some activity for the FSHR is still retained. Replacement of inactive (*S*)-THQ by the active (*R*)-THQ reduces the FSHR agonistic activity completely. 3) Antagonistic potencies on the FSHR for the hetero-dimeric compounds **(*R*)-7c-e** are enhanced compared to the homodimeric **(*R*,*R*)-4c-e** whereas mixing the recognition units (that is, monomeric ligands **3c** and **5c**) results in reduced antagonistic potencies. It would be of interest to further investigate if the LH/FSHR agonist that is present in **(*R*)-7c-e** has a positive (cooperativity) effect for the antagonistic potency of the (*R*)-THQ that is located at the other end of the spacer moiety.

Experimental procedures

Measurement of CRE-induced luciferase activity

Materials. Recombinant human LH (recLH) and human recombinant FSH (recFSH) were synthesized at Schering-Plough Research Institute, Oss, The Netherlands. Luclite® was obtained from Packard. All cell culture supplies were obtained from Gibco/BRL unless indicated otherwise. The human LH receptor cDNA[10] and human FSH receptor cDNA[11] were kindly provided by Dr. A.J.W. Hsueh, Stanford University.

Luciferase assay. Chinese Hamster Ovary (CHO)-K1 cells stably expressing the CRE-luciferase reporter with the human LH receptor or human FSH receptor were grown to 80-90% confluency in Dulbecco's MEM/Nutrient Mix F12 containing 5% bovine calf serum and supplemented with penicillin G (80 units/mL) and streptomycin (0.08 mg/mL) in 5% CO_2 at 37 °C. Cells were harvested using cell dissociation solution (Sigma). Aliquots of the cells were cryopreserved in DMSO without a loss of functional activity on LH receptor or FSH receptor.[12] On the day of the experiment, cells were thawed, washed with assay medium (Dulbecco's MEM/Nutrient Mix F12 supplemented with 1 mg/L bovine insulin (Sigma), 5 mg/L apo-transferrin (Sigma), penicillin G (80 units/mL) and streptomycin (0.08 mg/mL) and then resuspended in assay medium. The compounds were tested in quadruplicate at 10 concentrations ranging from final concentrations of 10 μM to 0.316 nM with half log intervals. In the agonistic assays, 10 μL of assay medium containing test compound and 3% DMSO, 10 μL of assay medium containing 3% DMSO with recLH (final concentration of 1 nM) or recFSH (final concentration of 586 pM) or 10 μL of assay medium containing 3% DMSO alone were added to the wells of a 384-well white culture plate followed by the addition of 10 μL of assay medium. Then, 10 μL of cell suspension containing 7,500 cells was added to the wells. The final concentration of DMSO was 1%. In case of antagonistic assays, 10 μL of test compound solution or 10 μL of assay medium alone were added to 10 μL of assay medium containing recLH (final concentration of 100 pM, EC_{80}) or recFSH (final concentration of 49 pM, EC_{80}). Then, 10 μL of cell suspension containing 7,500 cells was added to the wells. After incubation for 4 h in a humidified atmosphere in 5% CO_2 at 37°C, plates were allowed to adjust to room temperature for 1 h. Then, 15 μL of LucLite solution (Packard) was added to the incubation mixture. Following 60 min at room temperature in the dark, luciferase activity was measured in a Packard Topcount Microplate Scintillation and Luminescence Counter. Agonistic effects of the compounds were determined as percentage of the (maximal) effect induced by 1 nM recLH or 586 pM recFSH. Antagonistic effects of the compounds were expressed as percentage of the effect induced by 100 pM recLH or 49 pM recFSH. The EC_{50} or IC_{50} values (concentration of the test compound that elicits half-maximal (50 %) luciferase stimulation or inhibition compared to the compound's maximally attainable effect, respectively) and the efficacy values (maximal effect as percentage of the effect of recLH or recFSH) of the test compounds were determined using the software program MathIQ (version 2.0, ID Business Solutions Limited).

Chemical procedures

Reactions were executed at ambient temperatures unless stated otherwise. All solvents were removed by evaporation under reduced pressure. Reactions were monitored by TLC analysis using silica gel coated plates (0.2 mm thickness) and detection by 254 nm UV-light or by either spraying with a solution of $(NH_4)_6Mo_7O_{24} \times 4H_2O$ (25 g/L) or $(NH_4)_4Ce(SO_4)_4 \times 2H_2O$ (10 g/L) in 10% sulfuric acid followed by charring at ~150 °C. Column chromatography was performed on silica gel (40-63 μm). NMR spectra were recorded on a 600/150 MHz spectrometer. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Coupling constants (*J*) are given in Hz. All presented ^{13}C-APT spectra are proton decoupled. Where indicated, NMR peak assignments were made using COSY, NOESY (τ mix = 1 sec) and HMQC experiments. For LC-MS analysis, a HPLC-system (detection simultaneously at 214 and 254 nm) equipped with an analytical C_{18} column (4.6 mmD ×

250 mmL, 5µ particle size) in combination with buffers A: H_2O, B: CH_3CN and C: 1% aq TFA and coupled to a mass instrument with an electronspray interface (ESI) was used. For RP-HPLC purifications, an automated HPLC system equipped with a semi-preparative C_{18} column (5 µm C_{18}, 10Å, 150 × 21.2 mm) was used. The applied buffers were A: H_2O + ammonium acetate (20 mM) and B: CH_3CN. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 60000 at *m/z* 400 (mass range *m/z* = 150-2000) and dioctylpthalate (*m/z* = 391.28428) as a lock mass. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). Optical rotations were measured on a Propol automatic polarimeter (Sodium D-line, λ = 589 nm).

General procedure for the preparation of monomeric ligands (R)-7a-e and (S)-7a-e.
Monomeric compounds **(*R*)-3a**, **(*R*)-3b**, **(*R*)-3c**, **(*R*)-3d** or **(*R*)-3e** (12.5 µmol, prepared as described in Chapter 8) and **2** (7.3 mg, 15.0 µmol, prepared as described in Chapter 5) were dissolved in 10 mL DCM. The mixture was degassed under argon in an ultrasonic bath, until the volume was approximately 1.5 mL. Water and solutions of $CuSO_4$ and sodium ascorbate were degassed separately. Water (1.0 mL) and aqueous solutions of sodium ascorbate (50 µL, 1 eq) and $CuSO_4$ (50 µL, 20 mol%) were added. After stirring for 18 h, TLC and LC-MS showed all reactions to be completed. Reaction mixtures were concentrated and coevaporated with toluene (3x). The residue was dissolved in DCM and the product was isolated using silica column chromatography (1 to 3.5% MeOH in DCM). Pure products were obtained as yellow powdery crystals in 75-97% yield. R_f=0.45 (9% MeOH in DCM). Products with spacer length n=0 and n=1 were purified by semi-preparative HPLC (70 to 90% B).

Heterodimeric ligand (*R*)-7a. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.31 (s, 1H, N*H*CO), 7.96 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.72 (d, *J* = 8.4, 1H, CH Ar, THQ-8), 7.67 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.59 (d, *J* = 7.8, 3H, 3 × CH Ar), 7.45 (t, *J* = 7.8, 2H, 2 × CH Ar), 7.38 (t, *J* = 7.5, 1H, CH Ar), 7.27 (d, *J* = 7.8, 1H, CH Ar), 7.14 (d, *J* = 6.0, 2H, 2 × CH Trz), 7.10 (t, *J* = 5.4, 1H, N*H*CO), 6.98 (d, *J* = 9.0, 2H, 2 × CH Ar), 6.95 (d, *J* = 9.0, 1H, CH Ar, THQ-7), 6.88 (d, *J* = 7.2, 1H, CH Ar), 6.75 (d, *J* = 9.0, 2H, 2 × CH Ar), 6.67 – 6.65 (m, 2H, 2 × CH Ar), 5.92 (s, 2H, N*H*$_2$), 5.20 (s, 1H, N*H*CO), 5.07 (s, 2H, C*H*$_2$, OC*H*$_2$Ctrz), 4.85 – 4.81 (m, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 4.58 (t, *J* = 5.4, 1H, N*H*), 4.36 (s, 2H, C*H*$_2$, NHC*H*$_2$Ctrz), 3.76 (d, *J* = 5.4, 2H, C*H*$_2$, NHC*H*$_2$CONH), 2.60 (s, 3H, C*H*$_3$, SC*H*$_3$), 2.45 (d, *J* = 14.4, 1H, C*H*$_2$, THQ), 1.92 (s, 3H, C*H*$_3$, acetyl), 1.76 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.58 (s, 3H, C*H*$_3$(C-CH$_2$)), 1.41 (t, *J* = 12.6, 9H, 3 × CH$_3$, *t*-butyl), 1.33 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$), 1.21 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.2, 170.0, 169.6, 167.3, 165.5, 165.0 (4 × *C*=O, acetyl + 3 × amide + 2 × C$_q$ Ar), 156.4 (*C*$_q$(OCH$_2$Trz)), 147.2, 144.7, 144.5, 144.5, 141.8, 139.8, 137.7, 137.4, 135.8, 135.5, 135.6, 133.3 (12 × C$_q$ Ar), 130.0 (CH Ar), 129.0 (2 × CH Ar), 128.1 (3 × CH Ar), 127.6, 127.4, 127.2 (6 × CH Ar), 126.7 (CH Ar, THQ-7), 123.7, 123.1 (2 × CH Trz), 118.7, 118.6 (2 × CH Ar), 117.7 (C$_q$, H$_2$NC=*C*CO), 117.5 (CH Ar, THQ-10), 115.0 (CH Ar), 114.2 (2 × CH Ar), 112.6 (CH Ar), 96.6 (C$_q$ Ar), 61.8 (*C*H$_2$, O*C*H$_2$Ctrz), 58.5 (C$_q$ Ar, THQ-4), 56.2 (*C*H$_2$, THQ-3), 52.1 (C$_q$, *t*-butyl), 49.6, 49.5 (2 × *C*H$_2$, *C*H$_2$Ntrz), 48.0 (*C*H$_2$, NH*C*H$_2$CONH), 41.9 (C$_q$, *C*(CH$_3$)$_2$), 34.6 (*C*H$_2$, NH*C*H$_2$Ctrz), 29.4 (2 × *C*H$_3$, *C*H$_3$(C-CH$_2$) + C(*C*H$_3$)$_2$), 29.1 (3 × *C*H$_3$, *t*-butyl), 28.8 (*C*H$_3$, C(*C*H$_3$)$_2$), 25.5 (*C*H$_3$, acetyl), 14.4 (*C*H$_3$, *C*H$_3$S). [α]$_D^{20}$: 149° (c=0.34, $CHCl_3$). ESI-MS *m/z*: obsd 1137.47 $[M + H]^+$. HRMS *m/z* calcd for $C_{61}H_{64}N_{14}O_5S_2 + H^+$: 1137.46983, obsd 1137.47217; calcd for $C_{61}H_{64}N_{14}O_5S_2 + 2H^+$: 569.23855, 569.23828.

Heterodimeric ligand (*R*)-7b. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.35 (s, 1H, N*H*CO), 7.99 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.76 (d, *J* = 7.2, 1H, CH Ar, THQ-8), 7.70 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.62 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.54 (s, 1H, CH Ar, THQ-10), 7.47 (t, *J* = 7.5, 4H, 3 × CH Ar), 7.40 (t, *J* = 7.2, 1H, CH Ar), 7.33 (s, 1H, CH Trz), 7.24 (d, *J* = 7.8, 1H, CH Ar), 6.99 (d, *J* = 9.0, 2H, 2 × CH Ar), 6.97 (d, *J* = 9.0, 1H, CH Ar, THQ-7), 6.86 (d, *J* = 7.2, 1H, CH Ar),

6.82 (d, *J* = 9.0, 2H, 2 × CH Ar), 6.72-6.70 (m, 2H, 2 × CH Ar), 5.99 (s, 2H, N*H*$_2$), 5.21 (s, 1H, N*H*CO), 5.12 (s, 2H, C*H*$_2$, OC*H*$_2$Ctrz), 4.98 (t, *J* = 5.4, 1H, N*H*), 4.44 – 4.43 (m, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 4.39 (q, *J* = 4.8, 2H, C*H*$_2$, NHC*H*$_2$Ctrz), 3.84 (d, *J* = 4.2, 2H, C*H*$_2$, NHC*H*$_2$CONH), 3.74 – 3.71 (m, 4H, 2 × C*H*$_2$, C*H*$_2$OC*H*$_2$), 2.61 (s, 3H, C*H*$_3$, SC*H*$_3$), 2.46 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.94 (s, 3H, C*H*$_3$, acetyl), 1.78 (d, *J* = 14.4, 1H, C*H*$_2$, THQ), 1.58 (s, 3H, C*H*$_3$(C-CH$_2$)), 1.43 (t, *J* = 12.0, 9H, 3 × CH$_3$, *t*-butyl), 1.32 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$), 1.23 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.2, 170.3, 169.6, 167.2, 165.6, 165.1, 162.9 (4 × *C*=O, acetyl + 3 × amide + 2 × C$_q$ Ar), 156.4 (*C*$_q$(OCH$_2$Trz)), 147.6, 144.8, 144.7, 143.9, 141.8, 139.8, 137.8, 137.4, 135.8, 135.7, 133.4 (12 × C$_q$ Ar), 129.9 (CH Ar), 129.0 (2 × CH Ar), 128.2 (3 × CH Ar), 127.7, 127.4, 127.2 (6 × CH Ar), 126.7 (CH Ar, THQ-7), 123.7, 123.0 (2 × CH Trz), 118.7, 118.1 (2 × CH Ar), 117.7 (C$_q$, H$_2$NC=*C*CO), 117.4 (CH Ar, THQ-10), 114.9 (CH Ar), 114.3 (2 × CH Ar), 112.6 (CH Ar), 96.4 (C$_q$ Ar), 69.2 (2 × *C*H$_2$, *C*H$_2$O*C*H$_2$), 61.7 (*C*H$_2$, O*C*H$_2$CTrz), 58.5 (C$_q$, THQ-4), 56.2 (*C*H$_2$, THQ-3), 52.1 (C$_q$, *t*-butyl), 50.1, 50.0 (2 × *C*H$_2$, *C*H$_2$Ntrz), 47.8 (*C*H$_2$, NH*C*H$_2$CONH), 41.9 (C$_q$, *C*(CH$_3$)$_2$), 34.9 (*C*H$_2$, NH*C*H$_2$Ctrz), 29.4 (2 × *C*H$_3$, *C*H$_3$(C-CH$_2$) + C(*C*H$_3$)$_2$), 29.1 (3 × *C*H$_3$, *t*-butyl), 28.9 (*C*H$_3$, C(*C*H$_3$)$_2$), 25.6 (*C*H$_3$, acetyl), 14.4 (*C*H$_3$, *C*H$_3$S). $[\alpha]_D^{20}$: 141° (c=0.40, $CHCl_3$). ESI-MS *m/z*: obsd 1181.47 $[M + H]^+$. HRMS *m/z* calcd for $C_{63}H_{68}N_{14}O_6S_2 + H^+$: 1181.49604, obsd 1181.50110; calcd for $C_{63}H_{68}N_{14}O_6S_2 + 2H^+$: 591.25166, obsd 591.25146.

Heterodimeric ligand (*R*)-7c. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.50 (s, 1H, N*H*CO), 7.98 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.75 (s, 1H, CH Trz), 7.74 (s, 1H, CH Ar, THQ-8), 7.67 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.60 (d, *J* = 7.2, 2H, 2 × CH Ar), 7.58 (s, 1H, CH Ar, THQ-10), 7.50 (s, 1H, CH Trz), 7.46 (t, *J* = 7.5, 3H, 2 × CH Ar+ N*H*CO), 7.39 (t, *J* = 7.2, 1H, CH Ar), 7.24 (d, *J* = 7.8, 1H, CH Ar), 6.97 (d, *J* = 8.4, 2H, 2 × CH Ar), 6.94 (d, *J* = 8.4, 1H, CH Ar, THQ-7), 6.86 (d, *J* = 7.2, 1H, CH Ar), 6.79 (d, *J* = 8.4, 2H, 2 × CH Ar), 6.70 – 6.67 (m, 2H, 2 × CH Ar), 5.97 (s, 2H, N*H*$_2$), 5.22 (s, 1H, N*H*CO), 5.11 (s, 2H, C*H*$_2$, OC*H*$_2$Ctrz), 4.88 (s, 1H, N*H*), 4.48 – 4.46 (m, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 4.42 – 4.35 (m, 2H, C*H*$_2$, NHC*H*$_2$Ctrz), 3.78 (s, 2H, C*H*$_2$, NHC*H*$_2$CONH), 3.76 – 3.69 (m, 4H, 2 × C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.49 – 3.46 (m, 4H, 2 × C*H*$_2$, OC*H*$_2$C*H*$_2$O), 2.61 (s, 3H, C*H*$_3$, SC*H*$_3$), 2.45 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.92 (s, 3H, C*H*$_3$, acetyl), 1.77 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.57 (s, 3H, C*H*$_3$(C-CH$_2$)), 1.42 (s, 3 × CH$_3$, *t*-butyl), 1.34 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$), 1.23 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.2, 170.2, 169.6, 167.2, 165.6, 165.0, 162.8 (4 × *C*=O, acetyl + 3 × amide + 2 × C$_q$ Ar), 156.5 (*C*$_q$(OCH$_2$Trz)), 147.5, 144.7, 144.6, 144.4, 143.9, 141.7, 139.8, 137.6, 137.4, 135.7, 133.4 (12 × C$_q$ Ar), 129.9 (CH Ar), 129.0 (2 × CH Ar), 128.1, 128.1 (3 × CH Ar), 127.7, 127.3, 127.2 (6 × CH Ar), 126.7 (CH Ar, THQ-7), 124.1, 123.0 (2 × CH Trz), 118.7, 118.3 (2 × CH Ar), 117.7 (C$_q$, H$_2$NC=*C*CO), 117.5 (CH Ar, THQ-10), 114.9 (CH Ar), 114.1 (2 × CH Ar), 112.6 (CH Ar), 96.5 (C$_q$ Ar), 70.4, 70.6 (2 × *C*H$_2$, OC*H*$_2$C*H*$_2$O), 69.2, 69.2 (2 × *C*H$_2$, NtrzCH$_2$C*H*$_2$O), 61.8 (*C*H$_2$, O*C*H$_2$Ctrz), 58.5 (C$_q$, THQ-4), 56.1 (*C*H$_2$, THQ-3), 52.0 (C$_q$, *t*-butyl), 50.3, 50.1 (2 × *C*H$_2$, *C*H$_2$Ntrz), 47.8 (*C*H$_2$, NH*C*H$_2$CONH), 41.8 (C$_q$, *C*(CH$_3$)$_2$), 34.8 (*C*H$_2$, NH*C*H$_2$Ctrz), 29.4 (2 × *C*H$_3$, *C*H$_3$(C-CH$_2$) + C(*C*H$_3$)$_2$), 29.1 (3 × *C*H$_3$, *t*-butyl), 28.8 (*C*H$_3$, C(*C*H$_3$)$_2$), 25.5 (*C*H$_3$, acetyl), 14.4 (*C*H$_3$, *C*H$_3$S). $[\alpha]_D^{20}$: 162° (c=0.26, $CHCl_3$). ESI-MS *m/z*: obsd 1225.6 $[M + H]^+$. HRMS *m/z* calcd for $C_{65}H_{72}N_{14}O_7S_2 + H^+$: 1225.52226, obsd 1225.52209; calcd for $C_{65}H_{72}N_{14}O_7S_2 + 2H^+$: 613.26477, obsd 613.26471.

Heterodimeric ligand (*R*)-7d. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.48 (s, 1H, N*H*CO), 7.98 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.80 (s, 1H, CH Trz), 7.75 (d, *J* = 8.4, 1H, CH Ar, THQ-8), 7.67 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.60 (d, *J* = 6.6, 3H, 3 × CH Ar), 7.55 (s, 1H, CH Trz), 7.46 (t, *J* = 7.5, 2H, 2 × CH Ar), 7.39 (t, *J* = 7.2, 1H, CH Ar), 7.29 (s, 1H, N*H*CO), 7.24 (s, 1H, CH Ar), 6.99 (d, *J* = 9.0, 2H, 2 × CH Ar), 6.95 (d, *J* = 8.4, 1H, CH Ar, THQ-7), 6.88 (d, *J* = 7.2, 1H, CH Ar), 6.80 (d, *J* = 8.4, 2H, 2 × CH Ar), 6.70 – 6.68 (m, 2H, 2 × CH Ar), 5.98 (s, 2H, N*H*$_2$), 5.22 (s, 1H, N*H*CO), 5.08 (s, 2H, C*H*$_2$, OC*H*$_2$Ctrz), 4.78 (s, 1H, N*H*), 4.50 (t, *J* = 4.8, 2H, C*H*$_2$, C*H*$_2$Ntrz), 4.46 (d, *J* = 6.0, 2H, C*H*$_2$, NHC*H*$_2$Ctrz), 4.40 (t, *J* = 4.8, 2H, C*H*$_2$, C*H*$_2$Ntrz), 3.83 (t, *J* = 4.8, 4.8, 2H, C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.77 (s, 2H, C*H*$_2$, NHC*H*$_2$CONH), 3.73 (s, 2H, C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.56 – 3.49 (m, 8H, 4 × C*H*$_2$, OC*H*$_2$C*H*$_2$O), 2.61 (s, 3H, C*H*$_3$, SC*H*$_3$), 2.46 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.93 (s, 3H, C*H*$_3$, acetyl), 1.77 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.58 (s,

3H, C*H*$_3$(C-CH$_2$)), 1.43 (s, 3 × CH$_3$, *t*-butyl), 1.34 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$), 1.22 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1, 170.1, 169.6, 167.2, 165.6, 165.0, 162.7 (4 × *C*=O, acetyl + 3 × amide + 2 × C$_q$ Ar), 156.6 (*C*$_q$(OCH$_2$Trz)), 147.5, 144.7, 144.6, 144.1, 143.8, 141.7, 139.7, 137.6, 137.4, 135.7, 135.7, 133.4 (12 × C$_q$ Ar), 129.9 (CH Ar), 128.9 (2 × CH Ar), 128.1, 128.1 (3 × CH Ar), 127.7, 127.3, 127.1 (6 × CH Ar), 126.6 (CH Ar, THQ-7), 124.1, 123.1 (2 × CH Trz), 118.6, 118.4 (2 × CH Ar), 117.7 (C$_q$, H$_2$NC=*C*CO), 117.5 (*C*Har, THQ-10), 114.8 (CH Ar), 114.1 (2 × CH Ar), 112.7 (CH Ar), 96.5 (C$_q$ Ar), 70.4, 70.4, 70.4, 70.3 (4 × *C*H$_2$, OC*H*$_2$*C*H$_2$O), 69.2, 69.2 (2 × *C*H$_2$, NtrzCH$_2$*C*H$_2$O), 61.8 (*C*H$_2$, OC*H*$_2$Ctrz), 58.5 (C$_q$, THQ-4), 56.1 (*C*H$_2$, THQ-3), 52.0 (C$_q$, *t*-butyl), 50.3, 50.1 (2 × *C*H$_2$, *C*H$_2$Ntrz), 47.9 (*C*H$_2$, NH*C*H$_2$CONH), 41.8 (C$_q$, *C*(CH$_3$)$_2$), 34.7 (*C*H$_2$, NH*C*H$_2$Ctrz), 29.6, 29.3 (2 × *C*H$_3$, *C*H$_3$(C-CH$_2$) + C(*C*H$_3$)$_2$), 29.1 (3 × *C*H$_3$, *t*-butyl), 28.7 (*C*H$_3$, C(*C*H$_3$)$_2$), 25.5 (*C*H$_3$, acetyl), 14.4 (*C*H$_3$, *C*H$_3$S). [α]$_D^{20}$: 156° (c=0.33, $CHCl_3$). ESI-MS *m/z*: obsd 1269.7 [M + H]$^+$. HRMS *m/z* calcd for $C_{67}H_{76}N_{14}O_8S_2$ + 2H$^+$: 635.27787, obsd 635.27777.

Heterodimeric ligand (*R*)-7e. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.44 (s, 1H, N*H*CO), 7.98 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.80 (s, 1H, CH Trz), 7.75 (d, *J* = 6.6, 1H, CH Ar, THQ-8), 7.68 (d, *J* = 7.8, 3H, 3 × CH Ar), 7.61 (d, *J* = 6.6, 3H, 3 × CH Ar), 7.46 (t, *J* = 7.5, 3H, 3 × CH Ar), 7.39 (t, *J* = 7.2, 1H, CH Ar), 7.27 (s, 1H, CH Ar), 6.99 (d, *J* = 8.4, 2H, 2 × CH Ar), 6.95 (d, *J* = 8.4, 1H, CH Ar, THQ-7), 6.89 (d, *J* = 7.8, 1H, CH Ar), 6.81 (d, *J* = 8.4, 2H, 2 × CH Ar), 6.72 – 6.69 (m, 2H, 2 × CH Ar), 5.98 (s, 2H, N*H*$_2$), 5.22 (s, 1H, N*H*CO), 5.08 (s, 2H, C*H*$_2$, OC*H*$_2$Ctrz), 4.78 (s, 1H, N*H*), 4.50 (t, *J* = 5.1, 2H, C*H*$_2$, C*H*$_2$Ntrz), 4.47 (d, *J* = 5.4, 2H, C*H*$_2$, NHC*H*$_2$Ctrz), 4.42 (t, *J* = 4.1, 2H, C*H*$_2$, C*H*$_2$Ntrz), 3.83 (t, *J* = 4.8, 2H, C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.79 (s, 2H, C*H*$_2$, NHC*H*$_2$CONH), 3.75 (t, *J* = 5.1, 2H, C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.56 – 3.50 (m, 12H, 6 × C*H*$_2$, OC*H*$_2$C*H*$_2$O), 2.61 (s, 3H, C*H*$_3$, SC*H*$_3$), 2.47 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.93 (s, 3H, C*H*$_3$, acetyl), 1.77 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.59 (s, 3H, C*H*$_3$(C-CH$_2$)), 1.43 (s, 3 × CH$_3$, *t*-butyl), 1.34 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$), 1.22 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.2, 170.1, 169.6, 167.2, 165.6, 165.0, 162.7 (4 × *C*=O, acetyl + 3 × amide + 2 × C$_q$ Ar), 156.6 (*C*$_q$(OCH$_2$Trz)), 147.5, 144.71 144.6, 144.1, 143.8, 141.7, 139.7, 137.5, 137.4, 135.8, 135.6, 133.4 (12 × C$_q$ Ar), 129.9 (CH Ar), 129.0 (2 × CH Trz), 118.6, 118.5 (2 × CH Ar), 117.7 (C$_q$, H$_2$NC=*C*CO), 117.5 (CH Ar, THQ-10), 114.9 (CH Ar), 114.2 (2 × CH Ar), 112.8 (CH Ar), 96.5 (C$_q$ Ar), 70.5, 70.5, 70.3 (6 × *C*H$_2$, OC*H*$_2$*C*H$_2$O), 69.3, 69.3 (2 × *C*H$_2$, NtrzCH$_2$*C*H$_2$O), 61.8 (*C*H$_2$, OC*H*$_2$Ctrz), 58.5 (C$_q$, THQ-4), 56.1 (*C*H$_2$, THQ-3), 52.0 (C$_q$, *t*-butyl), 50.3, 50.2 (2 × *C*H$_2$, *C*H$_2$Ntrz), 47.9 (*C*H$_2$, NH*C*H$_2$CONH), 41.9 (C$_q$, *C*(CH$_3$)$_2$), 34.8 (*C*H$_2$, NH*C*H$_2$Ctrz), 29.7, 29.3 (2 × *C*H$_3$, *C*H$_3$(C-CH$_2$) + C(*C*H$_3$)$_2$), 29.1 (3 × *C*H$_3$, *t*-butyl), 28.7 (*C*H$_3$, C(*C*H$_3$)$_2$), 25.5 (*C*H$_3$, acetyl), 14.4 (*C*H$_3$, *C*H$_3$S). [α]$_D^{20}$: 134° (c=0.30, $CHCl_3$). ESI-MS *m/z*: obsd 1314.5 [M + H]$^+$. HRMS *m/z* calcd for $C_{69}H_{80}N_{14}O_9S_2$ + 2H$^+$: 657.29098, obsd 657.29132.

Heterodimeric ligand (*S*)-7a. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.47 (s, 1H, N*H*CO), 7.97 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.73 (d, *J* = 6.6, 1H, CH Ar, THQ-8), 7.67 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.59 (d, *J* = 7.8, 3H, 3 × CH Ar), 7.46 (t, *J* = 7.5, 2H, 2 × CH Ar), 7.39 (t, *J* = 7.2, 1H, CH Ar), 7.23 (s, 2H, 2 × CH Trz), 7.19 (d, *J* = 3.6, 2H, CH Ar, N*H*CO), 6.98 (d, *J* = 8.4, 2H, 2 × CH Ar), 6.95 (d, *J* = 8.4, 1H, CH Ar, THQ-7), 6.86 (d, *J* = 7.2, 1H, CH Ar), 6.75 (d, *J* = 9.0, 2H, 2 × CH Ar), 6.66 (d, *J* = 7.8, 2H, 2 × CH Ar), 5.93 (s, 2H, N*H*$_2$), 5.23 (s, 1H, N*H*CO), 5.05 (s, 2H, C*H*$_2$, OC*H*$_2$Ctrz), 4.83 – 4.81 (m, 5H, 2 × C*H*$_2$, C*H*$_2$Ntrz + N*H*), 4.35 (d, *J* = 4.8, 2H, C*H*$_2$, NHC*H*$_2$Ctrz), 3.76 (s, 2H, C*H*$_2$, NHC*H*$_2$CONH), 2.60 (s, 3H, C*H*$_3$, SC*H*$_3$), 2.45 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.92 (s, 3H, C*H*$_3$, acetyl), 1.76 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.57 (s, 3H, C*H*$_3$(C-CH$_2$)), 1.42 (s, 9H, 3 × CH$_3$, *t*-butyl), 1.33 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$), 1.21 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.1, 170.1, 169.5, 167.2, 165.6, 165.0, 162.7 (4 × *C*=O, acetyl + 3 × amide + 2 × C$_q$ Ar), 156.3 (*C*$_q$(OCH$_2$Trz)), 147.3, 144.6, 144.6, 144.5, 144.3, 141.7, 139.7, 137.6, 137.3, 135.7, 133.3 (12 × C$_q$ Ar), 129.9 (CH Ar), 128.9 (2 × CH Ar), 128.1 (3 × CH Ar), 127.6, 127.3, 127.1 (6 × CH Ar), 126.6 (CH Ar, THQ-7), 123.7, 123.1 (2 × CH Trz), 118.6, 118.4 (2 × CH Ar), 117.7 (C$_q$, H$_2$NC=*C*CO), 117.5 (CH Ar, THQ-10), 114.9 (CH Ar), 114.1 (2 × CH Ar), 112.5 (CH Ar), 96.5 (C$_q$ Ar), 61.6 (*C*H$_2$, OC*H*$_2$Ctrz), 58.5 (C$_q$, THQ-4), 56.1 (*C*H$_2$, THQ-3), 52.0 (C$_q$, *t*-butyl), 49.5, 49.4 (2 × *C*H$_2$, *C*H$_2$Ntrz), 47.8 (*C*H$_2$, NHC*H*$_2$CONH), 41.8 (C$_q$, *C*(CH$_3$)$_2$), 34.5

(*C*H$_2$, NH*C*H$_2$Ctrz), 29.3 (2 × *C*H$_3$, *C*H$_3$(C-CH$_2$) + C(*C*H$_3$)$_2$), 29.0 (3 × *C*H$_3$, *t*-butyl), 28.8 (*C*H$_3$, C(*C*H$_3$)$_2$), 25.5 (*C*H$_3$, acetyl), 14.3 (*C*H$_3$, *C*H$_3$S). $[\alpha]_{D}^{20}$: -159° (c=0.29, CHCl$_3$). ESI-MS *m/z*: obsd 1137.7 $[M + H]^+$. HRMS *m/z* calcd for $C_{61}H_{64}N_{14}O_5S_2 + H^+$: 1137.46983, obsd 1137.47095; calcd for $C_{61}H_{64}N_{14}O_5S_2 + 2H^+$: 569.23855, obsd 569.2384.

Heterodimeric ligand (*S*)-7b. ^{1}H NMR (600 MHz, CDCl$_3$) δ 8.42 (s, 1H, N*H*CO), 7.99 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.76 (d, *J* = 6.6, 1H, CH Ar, THQ-8), 7.69 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.61 (d, *J* = 7.2, 2H, 2 × CH Ar), 7.54 (s, 1H, CH Ar, THQ-10), 7.50 – 7.45 (m, 4H, 3 × CH Ar + N*H*CO), 7.39 (t, *J* = 7.5, 1H, CH Ar), 7.33 (s, 1H, CH Trz), 7.24 (d, *J* = 7.8, 1H, CH Ar), 6.99 – 6.95 (m, 3H, 3 × CH Ar), 6.85 (d, *J* = 7.8, 1H, CH Ar), 6.81 (d, *J* = 9.0, 2H, 2 × CH Ar), 6.71 – 6.69 (m, 2H, 2 × CH Ar), 5.98 (s, 2H, N*H*$_2$), 5.22 (s, 1H, N*H*CO), 5.11 (s, 2H, C*H*$_2$, OC*H*$_2$Ctrz), 4.98 (s, 1H, N*H*), 4.43 (d, *J* = 5.4, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz), 4.38 (q, *J* = 4.5, 2H, C*H*$_2$, NHC*H*$_2$Ctrz), 3.83 (s, 2H, C*H*$_2$, NHC*H*$_2$CONH), 3.73 – 3.70 (m, 4H, 2 × C*H*$_2$, C*H*$_2$OC*H*$_2$), 2.62 (s, 3H, C*H*$_3$, SC*H*$_3$), 2.46 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.93 (s, 3H, C*H*$_3$, acetyl), 1.77 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.57 (s, 3H, C*H*$_3$(C-CH$_2$)), 1.42 (s, 9H, 3 × CH$_3$, *t*-butyl), 1.31 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$), 1.23 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$). ^{13}C NMR (150 MHz, CDCl$_3$) δ 171.2, 170.33 169.6, 167.2, 165.6, 165.0, 162.9 (4 × *C*=O, acetyl + 3 × amide + 2 × C$_q$ Ar), 156.4 (*C*$_q$(OCH$_2$Trz)), 147.6, 144.8, 144.7, 143.9, 141.8, 139.8, 137.7, 137.3, 135.7, 133.3 (12 × C$_q$ Ar), 129.8 (CH Ar), 128.9 (2 × CH Ar), 128.1 (3 × CH Ar), 127.7, 127.3, 127.2 (6 × CH Ar), 126.7 (CH Ar, THQ-7), 123.7, 123.0 (2 × CH Trz), 118.7, 118.1 (2 × CH Ar), 117.7 (C$_q$, H$_2$NC=*C*CO), 117.4 (CH Ar, THQ-10), 114.8 (CH Ar), 114.2 (2 × CH Ar), 112.6 (CH Ar), 96.4 (C$_q$ Ar), 69.1 (2 × *C*H$_2$, *C*H$_2$OCH$_2$), 61.6 (*C*H$_2$, OCH$_2$Ctrz), 58.5 (C$_q$, THQ-4), 56.1 (*C*H$_2$, THQ-3), 52.0 (C$_q$, *t*-butyl), 50.1, 50.0 (2 × *C*H$_2$, *C*H$_2$Ntrz), 47.7 (*C*H$_2$, NHCH$_2$CONH), 41.8 (C$_q$, *C*(CH$_3$)$_2$), 34.8 (*C*H$_2$, NH*C*H$_2$Ctrz), 29.4 (2 × *C*H$_3$, *C*H$_3$(C-CH$_2$) + C(*C*H$_3$)$_2$), 29.1 (3 × *C*H$_3$, *t*-butyl), 28.8 (*C*H$_3$, C(*C*H$_3$)$_2$), 25.5 (*C*H$_3$, acetyl), 14.4 (*C*H$_3$, *C*H$_3$S). $[\alpha]_{D}^{20}$: -146° (c=0.18, CHCl$_3$). ESI-MS *m/z*: obsd 1181.7 $[M + H]^+$. HRMS *m/z* calcd for $C_{63}H_{68}N_{14}O_6S_2 + 2H^+$: 591.25166, obsd 591.25159.

Heterodimeric ligand (*S*)-7c. ^{1}H NMR (600 MHz, CDCl$_3$) δ 8.46 (s, 1H, N*H*CO), 7.98 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.75 (s, 1H, CH Trz), 7.74 (s, 1H, CH Ar, THQ-8), 7.68 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.60 (d, *J* = 7.8, 2H, 2 × CH Ar), 7.58 (s, 1H, CH Ar, THQ-10), 7.49 (s, 1H, CH Trz), 7.46 (t, *J* = 7.5, 2H, 2 × CH Ar), 7.41 – 7.39 (m, 2H, CH Ar + N*H*CO), 7.24 (s,1H, CH Ar), 6.97 (d, *J* = 8.4, 2H, 2 × CH Ar), 6.95 (d, *J* = 8.4, 1H, CH Ar, THQ-7), 6.87 (s, 1H, CH Ar), 6.79 (d, *J* = 9.0, 2H, 2 × CH Ar), 6.70-6.67 (m, 2H, 2 × CH Ar), 5.98 (s, 2H, N*H*$_2$), 5.22 (s, 1H, N*H*CO), 5.12 (s, 2H, C*H*$_2$, OC*H*$_2$Ctrz), 4.88 (s, 1H, N*H*), 4.48-4.46 (m, 4H, 2 × C*H*$_2$, C*H*$_2$Ntrz+NHC*H*$_2$Ctrz), 4.39 – 4.38 (m, 2H, C*H*$_2$, C*H*$_2$Ntrz), 3.78 (s, 2H, C*H*$_2$, NHC*H*$_2$CONH), 3.76 – 3.70 (m, 4H, 2 × C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.49 – 3.47 (m, 4H, 2 × C*H*$_2$, OC*H*$_2$C*H*$_2$O), 2.60 (s, 3H, C*H*$_3$, SC*H*$_3$), 2.45 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.93 (s, 3H, C*H*$_3$, acetyl), 1.77 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.57 (s, 3H, C*H*$_3$(C-CH$_2$)), 1.42 (s, 3 × CH$_3$, *t*-butyl), 1.34 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$), 1.23 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$). ^{13}C NMR (150 MHz, CDCl$_3$) δ 171.1, 170.1, 169.5, 167.1, 165.5, 165.0, 162.7 (4 × *C*=O, acetyl + 3 × amide + 2 × C$_q$ Ar), 156.5 (*C*$_q$(OCH$_2$Trz)), 147.5, 144.7, 144.6, 144.3, 143.8, 141.7, 139.7, 137.6, 137.6, 137.3, 135.7, 133.3 (12 × C$_q$ Ar), 129.8 (CH Ar), 128.9 (2 × CH Ar), 128.1, 128.0 (3 × CH Ar), 127.8, 127.6, 127.3, 127.2, 127.1 (6 × CH Ar), 126.6 (CH Ar, THQ-7), 124.0, 123.0 (2 × CH Trz), 118.6, 118.2 (2 × CH Ar), 117.6 (C$_q$, H$_2$NC=*C*CO), 117.4 (CH Ar, THQ-10), 114.8 (CH Ar), 114.1 (2 × CH Ar), 112.6 (CH Ar), 96.4 (C$_q$ Ar), 70.4, 70.2 (2 × *C*H$_2$, OC*H*$_2$C*H*$_2$O), 69.1, 69.1 (2 × *C*H$_2$, NtrzCH$_2$C*H*$_2$O), 61.7 (*C*H$_2$, OCH$_2$Ctrz), 58.4 (C$_q$, THQ-4), 56.1 (*C*H$_2$, THQ-3), 52.0 (C$_q$, *t*-butyl), 50.3, 50.1 (2 × *C*H$_2$, *C*H$_2$Ntrz), 47.8 (*C*H$_2$, NH*C*H$_2$CONH), 41.8 (C$_q$, *C*(CH$_3$)$_2$), 34.7 (*C*H$_2$, NH*C*H$_2$Ctrz), 29.3 (2 × *C*H$_3$, *C*H$_3$(C-CH$_2$) + C(*C*H$_3$)$_2$), 29.0 (3 × *C*H$_3$, *t*-butyl), 28.7 (*C*H$_3$, C(*C*H$_3$)$_2$), 25.4 (*C*H$_3$, acetyl), 14.3 (*C*H$_3$, *C*H$_3$S). $[\alpha]_{D}^{20}$: -143° (c=0.26, CHCl$_3$). ESI-MS *m/z*: obsd 1225.7 $[M + H]^+$. HRMS *m/z* calcd for $C_{65}H_{72}N_{14}O_7S_2 + 2H^+$: 613.26477, obsd 613.26489.

Heterodimeric ligand (*S*)-7d. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.43 (s, 1H, N*H*CO), 7.98 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.80 (s, 1H, CH Trz), 7.75 (d, *J* = 6.6, 1H, CH Ar, THQ-8), 7.68 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.61 (d, *J* = 7.8, 3H, 3 × CH Ar), 7.55 (s, 1H, CH Trz), 7.46 (t, *J* = 7.5, 2H, 2 × CH Ar), 7.39 (t, *J* = 7.2, 1H, CH Ar), 7.28-7.24 (m, 2H, CH Ar + N*H*CO), 6.99 (d, *J* = 8.4, 2H, 2 × CH Ar), 6.96 (d, *J* = 8.4, 1H, CH Ar, THQ-7), 6.89 (d, *J* = 7.8, 1H, CH Ar), 6.80 (d, *J* = 8.4, 2H, 2 × CH Ar), 6.71 – 6.68 (m, 2H, 2 × CH Ar), 5.98 (s, 2H, N*H*$_2$), 5.22 (s, 1H, N*H*CO), 5.09 (s, 2H, C*H*$_2$, OC*H*$_2$Ctrz), 4.78 (s, 1H, N*H*), 4.51 (t, *J* = 4.8, 2H, C*H*$_2$, C*H*$_2$Ntrz), 4.46 (d, *J* = 5.4, 2H, C*H*$_2$, NHC*H*$_2$Ctrz), 4.40 (t, *J* = 5.1, 2H, C*H*$_2$, C*H*$_2$Ntrz), 3.84 (t, *J* = 5.1, 2H, C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.78 (s, 2H, C*H*$_2$, NHC*H*$_2$CONH), 3.73 (t, *J* = 6.3, 2H, C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.57 – 3.49 (m, 8H, 4 × C*H*$_2$, OC*H*$_2$C*H*$_2$O), 2.61 (s, 3H, C*H*$_3$, SC*H*$_3$), 2.46 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.94 (s, 3H, C*H*$_3$, acetyl), 1.78 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.59 (s, 3H, C*H*$_3$(C-CH$_2$)), 1.43 (s, 3 × CH$_3$, *t*-butyl), 1.35 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$), 1.22 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.2, 170.1, 169.6, 167.2, 165.6, 165.0, 162.7 (4 × *C*=O, acetyl + 3 × amide + 2 × C$_q$ Ar), 156.6 (*C*$_q$(OCH$_2$Trz)), 147.5, 144.7, 144.6, 144.1, 143.8, 141.7, 139.7, 137.6, 137.4, 135.7, 135.6, 133.4 (12 × C$_q$ Ar), 129.9 (CH Ar), 128.9 (2 × CH Ar), 128.1, 128.1 (3 × CH Ar), 127.7, 127.3, 127.2 (6 × CH Ar), 126.6 (CH Ar, THQ-7), 124.1, 123.1 (2 × CH Trz), 118.6, 118.4 (2 × CH Ar), 117.7 (C$_q$, H$_2$NC=*C*CO), 117.5 (CH Ar, THQ-10), 114.9 (CH Ar), 114.1 (2 × CH Ar), 112.8 (CH Ar), 96.5 (C$_q$ Ar), 70.5, 70.4, 70.4, 70.3 (4 × *C*H$_2$, OCH$_2$*C*H$_2$O), 69.3, 69.2 (2 × *C*H$_2$, NtrzCH$_2$*C*H$_2$O), 61.8 (*C*H$_2$, OCH$_2$Ctrz), 58.5 (C$_q$, THQ-4), 56.1 (*C*H$_2$, THQ-3), 52.0 (C$_q$, *t*-butyl), 50.3, 50.1 (2 × *C*H$_2$, *C*H$_2$Ntrz), 47.9 (*C*H$_2$, NH*C*H$_2$CONH), 41.8 (C$_q$, *C*(CH$_3$)$_2$), 34.7 (*C*H$_2$, NH*C*H$_2$Ctrz), 29.7, 29.3 (2 × *C*H$_3$, *C*H$_3$(C-CH$_2$) + C(*C*H$_3$)$_2$), 29.1 (3 × *C*H$_3$, *t*-butyl), 28.7 (*C*H$_3$, C(*C*H$_3$)$_2$), 25.5 (*C*H$_3$, acetyl), 14.4 (*C*H$_3$, *C*H$_3$S). $[\alpha]_D^{20}$: -144° (c=0.23, $CHCl_3$). ESI-MS *m/z*: obsd 1269.7 $[M + H]^+$. HRMS *m/z* calcd for $C_{67}H_{76}N_{14}O_8S_2 + 2H^+$: 635.27787, obsd 635.27802.

Heterodimeric ligand (*S*)-7e. ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.41 (s, 1H, N*H*CO), 7.98 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.81 (s, 1H, CH Trz), 7.75 (dd, *J* = 1.8, 8.4, 1H, CH Ar, THQ-8), 7.68 (d, *J* = 8.4, 2H, 2 × CH Ar), 7.61 (d, *J* = 6.0, 4H, 4 × CH Ar), 7.46 (t, *J* = 7.5, 3H, 2 × CH Ar+ N*H*CO), 7.39 (t, *J* = 7.2, 1H, CH Ar), 7.28 – 7.26 (m, 1H, CH Ar), 6.99 (d, *J* = 8.4, 2H, 2 × CH Ar), 6.96 (d, *J* = 8.4, 1H, CH Ar, THQ-7), 6.89 (d, *J* = 7.2, 1H, CH Ar), 6.81 (d, *J* = 9.0, 2H, 2 × CH Ar), 6.72 – 6.69 (m, 2H, 2 × CH Ar), 5.98 (s, 2H, N*H*$_2$), 5.22 (s, 1H, N*H*CO), 5.11 (s, 2H, C*H*$_2$, OC*H*$_2$Ctrz), 4.76 (s, 1H, N*H*), 4.50 (t, *J* = 5.1, 2H, C*H*$_2$, C*H*$_2$Ntrz), 4.48 (d, *J* = 5.4, 2H, C*H*$_2$, NHC*H*$_2$Ctrz), 4.42 (t, *J* = 5.1, 2H, C*H*$_2$, C*H*$_2$Ntrz), 3.83 (t, *J* = 5.1, 2H, C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.79 (s, 2H, C*H*$_2$, NHC*H*$_2$CONH), 3.76 (t, *J* = 5.1, 2H, C*H*$_2$, NtrzCH$_2$C*H*$_2$O), 3.56-3.48 (m, 12H, 6 × C*H*$_2$, OC*H*$_2$C*H*$_2$O), 2.61 (s, 3H, C*H*$_3$, SC*H*$_3$), 2.47 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.94 (s, 3H, C*H*$_3$, acetyl), 1.78 (d, *J* = 13.8, 1H, C*H*$_2$, THQ), 1.59 (s, 3H, C*H*$_3$(C-CH$_2$)), 1.43 (s, 3 × CH$_3$, *t*-butyl), 1.32 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$), 1.23 (s, 3H, C*H*$_3$, C(C*H*$_3$)$_2$). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.2, 170.1, 169.6, 167.2, 165.6, 165.01 162.7 (4 × *C*=O, acetyl + 3 × amide + 2 × C$_q$ Ar), 156.6 (*C*$_q$(OCH$_2$Trz)), 147.5, 144.7, 144.7 144.1, 143.8, 141.8, 139.7, 137.5, 137.4, 135.8, 135.6, 133.4 (12 × C$_q$ Ar), 129.9 (CH Ar), 129.0 (2 × CH Ar), 128.1, 128.1 (3 × CH Ar), 127.7, 127.3, 127.5 (6 × CH Ar), 126.7 (CH Ar, THQ-7), 124.1, 123.2 (2 × CH Trz), 118.6, 118.5 (2 × CH Ar), 117.7 (C$_q$, H$_2$NC=*C*CO), 117.5 (CH Ar, THQ-10), 114.9 (CH Ar), 114.2 (2 × CH Ar), 112.8 (CH Ar), 96.5 (C$_q$ Ar), 70.5, 70.5, 70.3 (6 × *C*H$_2$, OCH$_2$*C*H$_2$O), 69.3, 69.3 (2 × *C*H$_2$, NtrzCH$_2$*C*H$_2$O), 61.8 (*C*H$_2$, OCH$_2$Ctrz), 58.5 (C$_q$, THQ-4), 56.1 (*C*H$_2$, THQ-3), 52.0 (C$_q$, *t*-butyl), 50.3, 50.2 (2 × *C*H$_2$, *C*H$_2$Ntrz), 47.9 (*C*H$_2$, NH*C*H$_2$CONH), 41.9 (C$_q$, *C*(CH$_3$)$_2$), 34.8 (*C*H$_2$, NH*C*H$_2$Ctrz), 29.8, 29.4 (2 × *C*H$_3$, *C*H$_3$(C-CH$_2$) + C(*C*H$_3$)$_2$), 29.1 (3 × *C*H$_3$, *t*-butyl), 28.7 (*C*H$_3$, C(*C*H$_3$)$_2$), 25.5 (*C*H$_3$, acetyl), 14.4 (*C*H$_3$, *C*H$_3$S). $[\alpha]_D^{20}$: -143° (c=0.22, $CHCl_3$). ESI-MS *m/z*: obsd 1313.7 $[M + H]^+$. HRMS *m/z* calcd for $C_{69}H_{80}N_{14}O_9S_2 + 2H^+$: 657.29098 obsd 657.29071.

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Summary and future prospects

The concept that receptor dimerization is involved in certain GPCR signaling processes, offers a new perspective for medicinal sciences. The question can be raised whether hetero- or homodimerization of a receptor of interest is a prerequisite for functioning and if so, can it be used to control its bioactivity properties. This Thesis describes a ligand-based approach, in which two distinct pharmacophores are connected by a spacer system of variable nature, length and rigidity, to study the phenomenon of dimerization of GPCRs involved in reproduction. As discussed in **Chapter 1**, several examples of dimeric ligands that target receptor dimers have recently appeared in literature. Occasionally, these dimeric ligands have enhanced potency or selectivity for subtype receptors compared to the monomeric counterparts. Sometimes a change in the intrinsic activity of the parent compound is observed. In most cases, the mechanism of binding of dimeric ligands to GPCRs has not been established, thereby hampering the development of dimeric ligands with predictable pharmacological profiles.

The first part of the Thesis describes an approach to prepare sets of dimeric ligands that target the gonadotropin-releasing hormone receptor (GnRHR). **Chapter 2** describes a flexible strategy towards dimeric ligands in which the size of the linker entity and the attachment site to the parent ligand are simultaneously varied. The synthesis and biological evaluation of all compounds, including a set of monomeric reference compounds, revealed that modification of one side in the parent molecule is detrimental for functional antagonism. For other dimeric ligands with a permitted functionalization site, an increase in potency was observed, compared to the monomeric counterparts.

The influence of the linker system on the bioactivity of the dimeric ligands was investigated in **Chapter 3**. The linkers were derived from a benzene core that is substituted on various positions with an acetylene function. The binding affinities of the monomeric and dimeric compounds were in the same order of magnitude while a significantly different functional profile was observed. The monomers proved to be full antagonists for the GnRHR while the dimeric ligands displayed only marginal antagonism at high concentration. Additional experiments showed a reduction of the maximal effect of the peptide agonist triptorelin in the presence of a dimeric ligand. However, this reduction was also observed for a monomeric ligand. Recent reports on the mode of binding of various GnRHR antagonists detailed that different residues of the receptor are involved in binding of different classes of antagonists. It was further reported that some molecules that highly resemble the structure of the parent compound used in Chapter 2 and 3, are insurmountable antagonists for the GnRH receptor. Insurmountable antagonists generally have a long residence

time on the receptor. Such receptor kinetics would hamper the design of successful dimeric ligands. It would be of interest to investigate whether insurmountable antagonism is the origin of the observed reduced antagonism for the dimeric ligands described in chapters 2 and 3.

Chapter 4 describes the synthesis and biological evaluation of homodimeric GnRHR antagonists having a different parent ligand and the same linker system as used in Chapters 2 and 3. It was observed that the functional antagonism of the monomeric compounds was diminished while the dimeric compounds still exhibit functional antagonism. In addition, a set of heterodimeric compounds was prepared that incorporates two different parent ligands (that is, a ligand as used in Chapter 2 and 3 on one side and a ligand used in this Chapter on the other side of the spacer). It appeared that, for some ligands, the binding affinities were not in the same order of magnitude as the functional antagonistic potencies. Similar results were also observed in the previous Chapters. The most potent compounds described in this Chapter were dimeric ligands that are interconnected with one ethylene glycol spacer unit (n=1). This holds true for both homo-dimers and heterodimers. This spacer may thus possess the optimal length for interaction of both ligands with the GnRHR (or a dimer). The question remains whether the dimeric ligands bind to two distinct receptors or that an increased local concentration, attained upon monovalent binding of the dimeric ligands, is at the basis of the altered pharmacological properties. It would therefore be of interest to prepare a compound that contains a photo-crosslinking group, an affinity- and a fluorophore tag as depicted in Figure 1. Similar monomeric probes have been prepared by Nan and co-workers to study the localization and function of $GABA_B$ receptors in living cells.[1] In this example, the dimeric ligand would then form a covalent linkage with the receptor molecule(s) upon irradiation with UV-light. The biotin affinity tag can be used to isolate the cross-linked proteins in a purification step with streptavidin. Subsequent analysis of the fluorescent bands by SDS-PAGE gel would provide information whether one or two receptor molecules are connected.

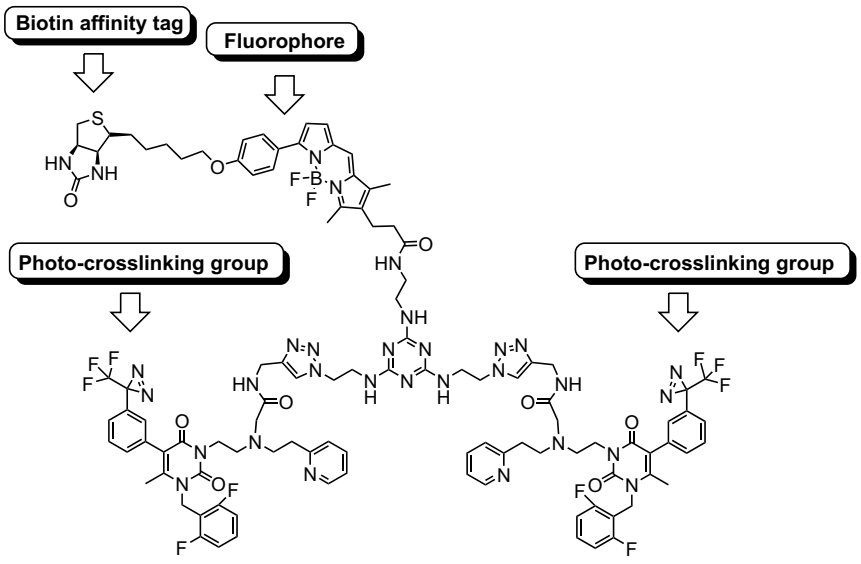

Figure 1. Design of a dimeric ligand that contains a fluorescent tag and two photo-crosslinking groups to explore whether one or two receptor molecules are involved in binding.

The second part of this Thesis focuses on the development of dimeric ligands for the glycoprotein hormone receptors (GpHRs), namely the luteinizing hormone receptor (LHR) and the follicle-stimulating hormone receptor (FSHR). As described in the introduction (Chapter 1), the GpHRs normally bind to a ~30 kDa large glycoprotein hormone. Most effective small molecule GpHR modulators described to date are thought to exert their biological activity by binding to the seven-helical transmembrane region.

Chapter 5 deals with the development of dimeric ligands that were derived from a low molecular weight LHR agonist. This ligand also shows activity on the FSHR, albeit with a lower potency. Two series of dimeric ligands having either rigid, benzene substituted spacers or more flexible polyethylene glycol spacers, were prepared. Biological evaluation of the dimeric ligands led to the discovery of more selective LHR agonists compared to FSHR. Either a decrease in potency or a drop in efficacy on the FSHR caused the enhanced selectivity of the dimeric ligands compared to the monomeric counterparts. Based on these encouraging results, it would be of interest to further investigate the origin of the selectivity for GpHRs in more detail.

Some of the linker systems of the dimeric ligands used in Chapters 3 and 5 possess a proline motif. This motif was recognized as a scaffold molecule that could be obtained by a novel Staudinger/aza-Wittig/Ugi multi component reaction (SAWU-MCR). The SAWU-MCR was previously used on carbohydrate derived azido-aldehydes to obtain oligo-hydroxyl substituted proline derivatives. **Chapter 6** describes the use of this MCR to obtain dimeric ligands that are rigid in nature, but due to the multiple hydroxyl functions are expected to show improved solubility properties. The newly formed stereo-centers of the compounds appear to be in a 2,3-*cis* relation, which is in agreement with the previously reported stereogenic outcome using D-*lyxo*-pyrroline in the SAWU-3CR.[2,3] The monomeric compound prepared in this fashion possesses similar LHR and FSHR agonistic activities as was observed with the compounds that lack the hydroxyl functions. The *ortho*- and *para*-substituted compounds with the hydroxylated proline spacer show a significantly reduced activity (10- and 5-fold) on the LHR while the *meta*-dimer was two times more active than the corresponding monomer. In all cases, the dimeric compounds were more selective for the LHR than the monomeric compounds. This is in agreement with the results from the studies described in Chapter 5.

Chapter 7 describes the usage of an oligoproline (OP) linker for the development of dimeric ligands. It has been established that oligoprolines adopt a helical conformation and it was investigated whether such OP may be functionalized with the LH agonist (LHA) used in Chapters 5 and 6 without disturbing its helical conformation. It was envisioned that the proline helices may possess sufficient flexibility, necessary to allow interaction of the dimeric ligand with the receptors. In addition, some compounds were prepared and assayed that hold more than two ligands. For all synthesized compounds, a typical PP type II helix was evidenced by circular dichroism indicating that decoration of the helix with relatively large LHR agonists did not affect the helical conformation. Pharmacological evaluation revealed a significant increase in potency on the LHR that is related to the number of LHA incorporated. Apart from this, some compounds

exhibited an increase in selectivity for the LHR compared to the FSHR. These features indicate that oligoproline is a suitable linker for the development of dimeric or oligomeric ligands as probes to study the dimeric ligand effect to G-protein coupled receptors in more detail.

It would be of interest to investigate if the increase in potency that was observed for some compounds with multiple LHAs can be further enhanced by increasing the number of LHA. Recent reports on the adenosine receptor showed the use of a poly(amidoamine) (PAMAM) dendrimer that was decorated with an adenosine (A_{2A}) agonist.[4] PAMAM dendrimers are treelike macromolecules that, because of their biocompatibility, have found many applications in biomedicine.[5,6] The third generation dendrimer (G3), that contained 31 adenosine agonists and a fluorophore, exhibited improved pharmacological profiles compared to the monomeric agonist. In this report, the lower generation dendrimers (G0-G2) were not included and it would interesting to investigate the bioactivity of such molecules (depicted in Figure 2).

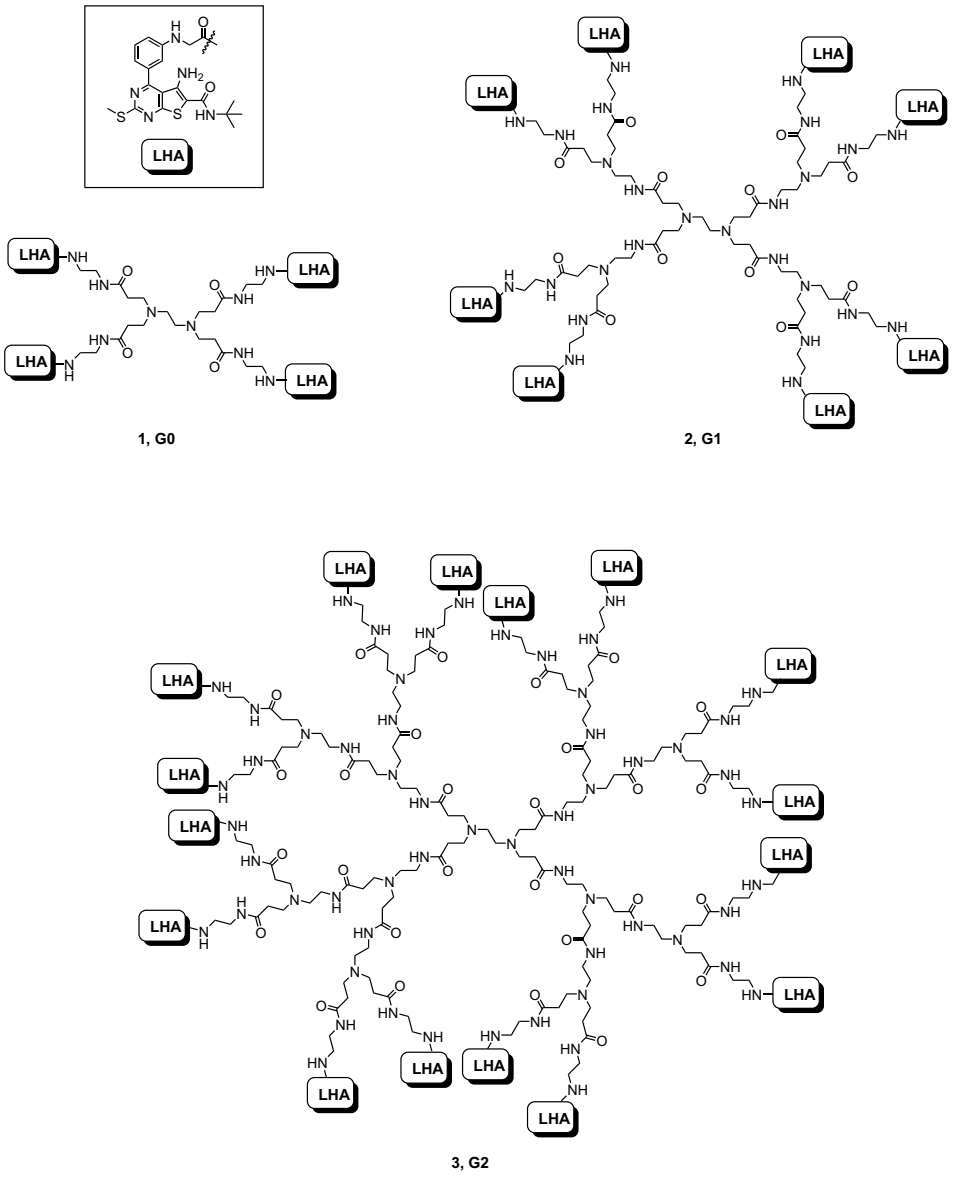

Figure 2. Higher oligomeric LHA ligands based on PAMAM dendrimers.

Although some of the dimeric compounds presented in Chapter 2-7 have other activity profiles in comparison with their monomeric counterparts, it is difficult to establish whether these ligands truly bind to two distinct receptor molecules. **Chapter 8** describes the use of a FSH pharmacophore with a chiral center. It was previously established that one of the enantiomers is a potent antagonist while the other is inactive on the FSH receptor. The described results indicate the potential of dimeric ligands provided with one active and one inactive enantiomer, as valuable tools to explore the mode of binding of dimeric ligands to the receptor (dimer). The dimeric ligands having active enantiomers on either side of the spacer show an increase in activity upon increasing spacer length. When at one side of the linker an active ligand is replaced by an inactive enantiomer, the antagonistic potency decreases with increasing spacer length. This demonstrates that the second pharmacophore contributes to the observed antagonistic potency on the FSHR.

Finally, the nature of the interaction of the dimeric ligands with the FSH receptor was investigated with a set of dimeric ligands that are composed of an antagonist on one side and an agonist on the other side of the linker. **Chapter 9** describes the synthesis and biological evaluation of such 'hetero'-dimeric compounds. To further explore the binding mode of the ligands, the separate ligands were also mixed (instead of covalently linked) and biologically evaluated. Interconnecting an FSHR antagonist to a LH/FSHR agonist provided compounds with unique pharmacological properties. For example, no FSHR agonistic activities were observed for these hetero-dimeric compounds, while the compounds were potent agonists on the LHR. This effect could not be obtained by solely mixing the separate recognition units. It was also observed that antagonistic potencies of the heterodimeric compounds were enhanced for the FSHR compared to the homodimeric antagonists described in Chapter 8, whereas mixing the monomeric recognition units resulted in reduced antagonistic potencies. It would be of interest to investigate if the LH/FSHR agonist that is present in the heterodimeric compounds has a positive (cooperativity) effect on the potency of the FSHR antagonist that is located at the other end of the spacer moiety.

The research in this Thesis was focused on mechanistic understanding rather than identification of drug development candidates. It is evident that the dimeric ligands described in this Thesis will exhibit physicochemical characteristics (for example, log P, MW, polar surface area) that are unfavorable for oral absorption (described as Lipinski rule of five, Box 1).[7-9] Dimeric ligands are large compounds with an average molecular weight of about 1300 and some were estimated to have a log P of 7-8. One way to circumvent these high non-druglike properties is to develop a set of molecules with linker molecules that can assemble in a non-covalent manner. The luteinizing hormone receptor agonist **1** may be functionalized with a 2-ureido-4[1H]-pyrimidinone unit (also called 'Upy', depicted in Figure 3).[10] Upy's are known to dimerize via strong hydrogen bonding with an association constant (K_a) of 1 x 10^7 in chloroform that is saturated with water.[10] Although the K_a is probably less favorable in pure water, studies utilizing Upy's evidenced that dimerization still took place in this H-bond rich environment.[11]

Box 1: Lipinki rule of five

More than 50% of the FDA approved drugs fulfill Lipinski´s rule of five. This rule describes four molecular properties for efficient intestinal absorption. These are 1) the molecule has a weight below 500 g/mole; 2) its calculated log P is lower than 5; 3) it possesses less than 5 H-bond donors and 4) it possesses less than 10 H-bond acceptors.

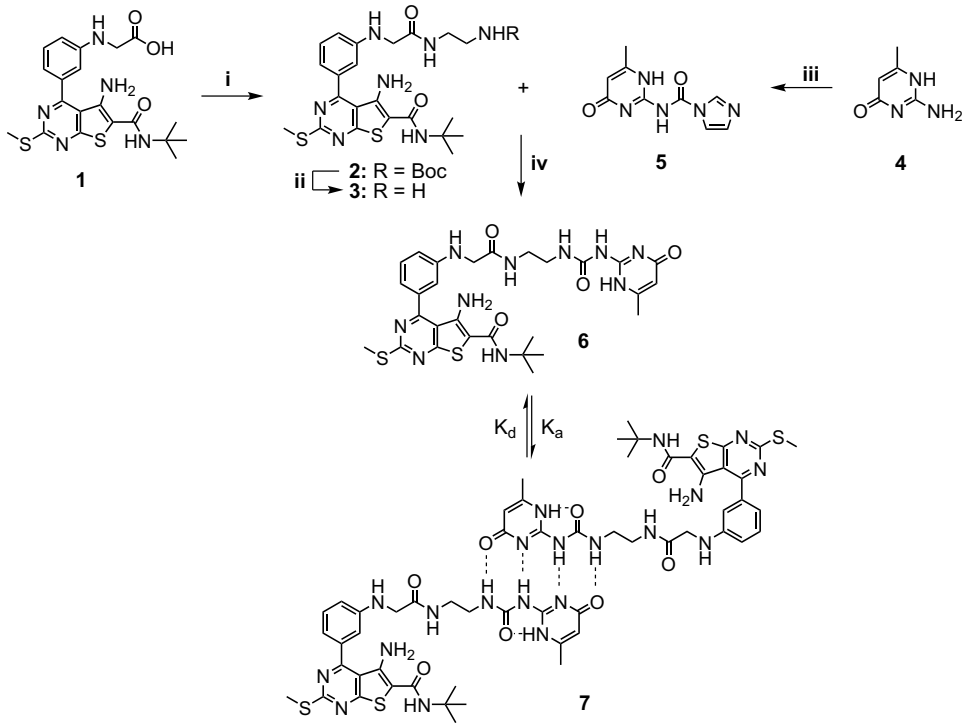

Figure 3. Development of dimeric ligands with self-organizing molecules as linker system. *Reagents and conditions*: *i*. *N*-Bocethylenediamine, BOP, DiPEA, DMF; *ii*. TFA/DCM; *iii*. CDI, $CHCl_3$; *iv*. Et_3N, $CHCl_3$.

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Samenvatting

G-eiwit gekoppelde receptoren (GPCRs) zijn membraan eiwitten welke gekarakteriseerd worden door zeven transmembraan helices. De functie van GPCRs is het overbrengen van een signaal door het celmembraan van extra-cellulaire liganden welke uiteen lopen van fotonen, ionen, kleine eiwitten tot hormonen. GPCRs zijn betrokken bij veel biologische processen en het is dan ook niet verwonderlijk dat deze eiwitfamilie intensief bestudeerd wordt.

In de afgelopen twee decennia is er veel literatuur verschenen over the betrokkenheid van receptor dimerisatie in het functioneren en het bestaan van GPCRs. Een voor de hand liggend gevolg hierop is dat verscheidene onderzoeksgroepen ook liganden zijn gaan ontwikkelen welke op deze dimere receptoren werken. Sommige van deze dimere liganden, waarin twee farmacophoren zijn verbonden door middel van een linker, blijken een verbeterde biologische activiteit te bezitten. In enkele gevallen is een verhoogde affiniteit en selectiviteit voor subklasse receptoren vernomen en sommige liganden blijken een andere werking te hebben als ze verbonden worden. In de meeste gevallen is het niet duidelijk waardoor deze dimere liganden een andere of verbeterde bioactiviteit geven en dit remt de ontwikkeling van dimere liganden met een voorspelbare farmacologisch profiel. Het onderzoek beschreven in dit proefschrift is gericht op de synthese en de biologische evaluatie van dimere liganden voor receptoren welke betrokken zijn bij de humane reproductie. Voor deze receptoren is ook beschreven dat dimerisatie een rol speelt in het bestaan en functioneren ervan. **Hoofdstuk 1** geeft een uitgebreid overzicht over de betrokken aspecten van receptor dimerisatie. Ook worden er voorbeelden gegeven van dimere liganden welke eerder beschreven zijn voor andere GPCRs.

Het eerste deel van dit proefschrift beschrijft een benadering om een set van dimere liganden te ontwikkelen welke interactie hebben met de gonadotropin stimulerend hormoon receptor (GnRHR). **Hoofdstuk 2** beschrijft een strategie naar de synthese van dimere liganden welke flexibel is wat betreft de lengte van de linker en het aanhechtingspunt van de moederverbinding. De biologische evaluatie van de verbindingen, inclusief een paar monomere referentiemoleculen, liet zien dat modificatie aan een kant van de moederverbinding de antagonistische werking verstoorde. Voor sommige dimere liganden waar de functionalisatie geen dramatisch effect had op de antagonistische werking, werd een verhoogde potentie waargenomen vergeleken met de overeenkomstige monomere liganden.

De invloed van de aard van de linker op de activiteit van de dimere liganden werd onderzocht in **Hoofdstuk 3**. De linkers werden vervaardigd uit een benzeen ring welke op verschillende posities gesubstitueerd is met een acetyleen. De bindingsaffiniteit van de monomere en dimere

liganden waren in dezelfde orde van grootte terwijl het functionele profiel varieerde. De monomere liganden bleken volledige antagonisten voor de GnRHR te zijn terwijl de dimere liganden een lage antagonistische activiteit bezaten bij de hoogste testconcentratie. Additionele experimenten onthulde dat de dimere liganden het effect van de peptide agonist, triptorelin konden verlagen. Dit effect werd ook, tegen verwachting in, waargenomen voor de monomere liganden. Recent gepubliceerde artikelen over de bindingsprofielen van verscheidene GnRHR antagonisten beschreven dat verschillende residuen van de receptor betrokken zijn bij de binding van de verschillende antagonisten. Er is ook gerapporteerd dat een verbinding welk sterk op de in Hoofdstuk 2 en 3 gebruikte antagonist lijkt een zogenoemde 'onoverwinnelijke' antagonist is voor de GnRHR. Deze onoverwinnelijke antagonisten hebben over het algemeen een lange interactietijd met de receptor. Het zou interessant zijn verder te onderzoeken of het onoverwinnelijke antagonistische effect de reden is voor de waargenomen gereduceerde antagonistische activiteit van de dimere liganden welke gebruikt zijn in Hoofdstukken 2 en 3.
Hoofdstuk 4 beschrijft de synthese en de biologische evaluatie van homodimere GnRHR antagonisten welke afgeleid zijn van een andere moederverbinding maar gebaseerd op hetzelfde linkersysteem als gebruikt in Hoofdstuk 2. Het blijkt dat de monomere liganden nauwelijks antagonistische activiteit vertoonden bij de hoogst gemeten testconcentratie van 10 μM. De dimere liganden bleken volledige antagonisten te zijn. Er is ook een set van heterodimere liganden gesynthetiseerd die twee verschillende liganden bezitten (één ligand dat gebruikt is in Hoofdstuk 2 en 3 en één ligand dat gebruikt is in dit Hoofdstuk). Het blijkt dat de bindingsaffiniteit van sommige liganden niet in dezelfde orde van grootte is als hun functionele antagonistische potentie. Dit resultaat was ook vernomen in de voorgaande hoofdstukken. De meest potente verbinding beschreven in dit hoofdstuk zijn dimere liganden met één ethyleen glycol deel (n = 1). Dit geldt voor de homodimeren zowel als de heterodimeren met twee verschillende liganden. Deze linkerlengte zou dus de optimale lengte kunnen bezitten voor de interactie van beide liganden met de receptor (dimeer).

Het tweede deel van dit Proefschrift beschrijft de ontwikkeling van dimere liganden voor gonadotropin receptoren zoals de luteïniserend hormoon receptor (LHR) en de follikel stimulerend hormoon receptor (FSHR). Zoals beschreven in Hoofdstuk 1 binden deze receptoren normaliter met de N-terminus aan een ~30 kDa groot hormoon. Vanuit een therapeutisch oogpunt is de mogelijkheid om een bepaalde GPCR te moduleren met een klein molecuul een grote importantie. De meeste kleine moleculen die een werking hebben op de gonadotropin receptoren blijken een interactie te hebben met het transmembrane gedeelte van de receptor.
Hoofstuk 5 beschrijft de ontwikkeling van dimere liganden die afgeleid zijn van een laag moleculaire LHR agonist. Dit ligand blijkt verassend genoeg ook een werking te hebben op de FSHR, ook al is dat met een lagere potentie. Twee series van dimere liganden zijn gesynthetiseerd die afgeleid zijn van de benzeen gesubstitueerde linkers (gebruikt in Hoofdstuk 3) of van de meer flexibelere polyethyleen glycol linkers (gebruikt in Hoofdstuk 2 en 4). Biologische evaluatie van de

dimere liganden leidde tot de ontdekking van selectievere LHR agonisten vergeleken tot de FSHR. De selectiviteit werd verkregen door een vermindering in de potentie of in de effectiviteit voor de FSHR van de dimere liganden vergeleken met de monomere liganden. Het zou interessant zijn de oorsprong van deze selectiviteit verder te onderzoeken.

Sommige linker systemen van de dimere liganden gebruikt in Hoofdstukken 3 en 5 bevatten een proline structuur. Deze structuur werd herkend als een motief welke verkregen zou kunnen worden bij een recent ontwikkelde Staudinger aza-Wittig Ugi multi componenten reactie (SAWU-MCR). Deze MCR is eerder gebruikt op suiker afgeleide azido-aldehyden om oligohydroxy gesubstitueerde proline derivaten te verkrijgen. **Hoofdstuk 6** beschrijft het gebruik van deze MCR om dimere liganden te verkrijgen welke rigide van aard zijn maar door de meerdere hydroxy functionaliteiten, verbeterde oplosbaarheids eigenschappen kunnen bezitten. Het nieuwgevormde stereocentrum wat verkregen wordt in de moleculen blijkt een 2,3-cis relatie met het chirale buur-koolstof atoom te hebben. Dit komt overeen met de eerder gevonden uitkomst wanneer D-lyxo-pyrroline gebruikt wordt in de SAWU-3CR. Uit de biologische evaluatie van de verbindingen blijkt dat de agonistische activiteit voor de LHR en de FSHR vergelijkbaar zijn met de verbindingen die geen hydroxyl functies bevatten. De ortho- en para-gesubstitueerde verbindingen met de gehydroxyleerde proline linker bevatten een significant gereduceerde activiteit (10- en 5-voudig) voor de LHR terwijl de meta-dimeer twee keer zo actief was als de corresponderende monomeer. In alle gevallen bleken de dimere liganden een verhoogde selectiviteit te bevatten voor de LHR in vergelijking met monomere liganden. Dit komt overeen met de resultaten beschreven in Hoofdstuk 5.

Hoofdstuk 7 beschrijft het gebruik van een oligoproline (OP) linker in de ontwikkeling van dimere liganden voor de LHR. Oligoprolines nemen een helix conformatie aan. Er werd onderzocht of deze oligoprolines gefunctionaliseerd kunnen worden met een LH agonist (LHA) welke gebruikt werd in Hoofdstuk 5 en 6 zonder dat de conformatie van de helix verstoord wordt. Er werd aangenomen dat de proline helices nog voldoende flexibiliteit zouden bevatten voor een goede interactie met de receptor moleculen. Er werden ook een aantal verbindingen gesynthetiseerd welke drie of vier LHAs bevatten. Voor alle verbindingen werd een typische polyproline type II helix waargenomen met circulair dichroism wat aangeeft dat het functionaliseren van de helix met relatief grote moleculen de conformatie niet beïnvloedt. Biologische evaluatie van de verbindingen liet zien dat de potentie van de verbindingen toenam met het aantal LHA liganden (tot aan drie liganden). Sommige liganden bleken ook een verhoogde selectiviteit voor de LHR te bevatten in vergelijking tot de FSHR. Deze gegevens duiden erop dat oligoprolines geschikte verbindingen zijn om te gebruiken als linkers voor dimere en oligomere liganden.

Hoewel sommige van de dimere liganden uit Hoofdstukken 2-7 een andere bioactiviteit bevatten dan de corresponderende monomere liganden blijft het moeilijk vast te stellen of deze dimere liganden daadwerkelijk aan twee aparte receptoren binden. **Hoofdstuk 8** beschrijft het gebruik van een FSH ligand welke een chiraal koolstofatoom bevat. Het is beschreven dat een van deze

enantiomeren een potente antagonist is terwijl de andere enantiomeer inactief is op de FSHR. De resultaten beschreven in dit hoofdstuk wijzen op de potentie voor een dimeer ligand die bestaat uit een actieve en een inactieve enantiomeer als waardevol middel om de binding van de dimere liganden aan een receptor (dimeer) te onderzoeken. De dimere liganden die een actieve enantiomeer aan beide zijden van de linker bevatten blijken een toename in activiteit te bevatten evenredig met toename van de linker lengte. Wanneer aan één kant de actieve enantiomeer wordt vervangen door de inactieve neemt de activiteit af met toenemende lengte van de linker. Dit demonstreert dat de tweede pharmacofoor bijdraagt aan de antagonistische potentie en de binding van de liganden voor de FSHR.

Tenslotte wordt de aard van de interactie van de dimere liganden met de FSH receptor verder onderzocht met behulp van een set van liganden die bestaan uit een antagonist aan één kant en een agonist aan de andere kant van de linker. **Hoofdstuk 9** beschrijft de synthese en de biologische evaluatie van zulke heterodimere liganden. Om verder inzicht te krijgen in de binding van de liganden worden de beide liganden ook in mengvorm (in plaats van verbonden) getest. De heterodimere liganden die een FSH antagonist en een LH/FSHR agonist bevatten lieten unieke farmacologische eigenschappen zien. Er werd bijvoorbeeld geen FSHR agonistische activiteit gevonden voor deze heterodimere liganden, terwijl de verbindingen potente agonisten waren op de LHR. Dit effect kon niet worden verkregen wanneer de gescheiden liganden werden gemengd. Het werd ook duidelijk dat de antagonistische activiteit voor de heterodimere liganden hoger was voor de FSHR in vergelijking met de in Hoofdstuk 8 beschreven homodimere liganden. Wanneer de verbindingen gemengd werden werd een lagere antagonistische activiteit verkregen. Het zou interessant zijn te onderzoeken of de LH/FSHR agonist in de heterodimeer een positief (coöperatief) effect heeft op de antagonistische werking van de FSH ligand die aanwezig is aan de andere kant van de linker.

In een slothoofdstuk wordt een vooruitblik geworpen op toekomstige mogelijkheden om de vraag verder te beantwoorden hoe dimere liganden binden aan GPCRs.

List of publications

Kimberly M. Bonger, Sascha Hoogendoorn, Chris J. van Koppen, Cornelis M. Timmers, Herman S. Overkleeft, Gijsbert A. van der Marel. Synthesis and biological evaluation of heterodimeric LHR-FSHR ligands. *Manuscript in preparation.*

Kimberly M. Bonger, Sascha Hoogendoorn, Chris J. van Koppen, Cornelis M. Timmers, Herman S. Overkleeft, Gijsbert A. van der Marel. Synthesis and biological evaluation of dimeric FSHR antagonists. *Manuscript in preparation.*

Kimberly M. Bonger, Varsha Kapoerchan, Chris J. van Koppen, Cornelis M. Timmers, Herman S. Overkleeft, Gijsbert A. van der Marel. Oligoproline helix as a scaffold for potent, selective and structurally defined dimeric LHR ligands. *Manuscript in preparation.*

Kimberly M. Bonger, Richard J. B. H. N. van den Berg, Annemiek D. Knijnenburg, Laura H. Heitman, Chris J. van Koppen, Cornelis M. Timmers, Herman S. Overkleeft, Gijsbert A. van der Marel. Discovery of Selective Luteinizing Hormone Receptor Agonists by the bivalent ligand method. *Submitted for publication.*

Kimberly M. Bonger, Richard J. B. H. N. van den Berg, Laura H. Heitman, Ad P. IJzerman, Julia Oosterom, Cornelis M. Timmers, Herman S. Overkleeft, Gijsbert A. van der Marel. GnRHR binding and antagonism of dimeric systems appear dependent on the nature of the parent pharmacophore. *Submitted for publication.*

Kimberly M. Bonger, Tom Wennekes, Gerrit Lodder, Dmitri Filippov, Gijs A. van der Marel and Herman S. Overkleeft. The effect of Lewis Acids on the stereochemical outcome of L-Lyxo Pyrroline with the tandem Staudinger aza-Wittig Ugi 3CR. *Eur. J. Org. Chem.* **2008**, *21*, 3678-3688

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Curriculum Vitae

Kimberly Michelle Bonger werd op 2 november 1980 geboren te Toronto, Canada. Na het behalen van het HAVO-diploma aan het Herbert Vissers College te Nieuw-Vennep in 1997 werd in dat jaar begonnen met de studie Organische Chemie aan de Hogeschool Leiden. Van september 1999 tot mei 2000 werd in het kader van een afstudeerstage onderzoek gedaan naar de synthese van het natuurproduct Athanagrandione aan de *vrije* Universiteit *amsterdam* onder leiding van prof. dr. R. V. A. Orru. De opleiding werd in augustus 2001 met goed gevolg afgerond. Gedurende het derde jaar van de studie Organische Chemie werd tevens begonnen aan een doctoraal-instroomprogramma scheikunde aan de *vrije* Universiteit *amsterdam*. Van augustus 2001 tot maart 2002 werd in het kader van een hoofdvakstage onderzoek verricht aan de University of Stavanger, Noorwegen, onder leiding van dr. E. Bakstad naar de synthese van het natuurproduct Hazardiadione. Het doctoraal diploma werd behaald in augustus 2002.

Van januari 2004 tot december 2008 werd als Assistent in Opleiding het in dit proefschrift beschreven onderzoek uitgevoerd bij de vakgroep Bio-organische Synthese onder leiding van prof. dr. G. A. van der Marel en prof. dr. H. S. Overkleeft in samenwerking met dr. C. M. Timmers van het Schering-Plough research institute.

Delen van de in dit proefschrift vermeldde resultaten werden vertoond in een mondelinge presentatie op het “3rd international conference on Multi-Component Reactions and Related Chemistry” in juli 2006 te Amsterdam, het “International Symposium on Advances in Synthetic and Medicinal Chemistry” in augustus 2007 te St. Petersburg, Rusland en de bijeenkomst van de afdeling farmacochemie van de Koninklijke Nederlandse Chemie Vereniging (KNCV) in september 2007 te Lunteren. Voor de laatste twee bijeenkomsten is een prijs toegekend voor de beste mondelinge communicatie.

In de lente van 2009 zal als postdoc worden begonnen in de groep van prof. T. Wandless aan Stanford University te Palo Alto, Californië.

Nawoord

Promotieonderzoek kent veel uitersten en doe je nooit alleen. Zonder de hulp, steun en kennis van bepaalde, of eigenlijk een heleboel personen zou dit proefschrift van veel minder betekenis zijn geweest. Allereerst natuurlijk mijn ouders; Ik ga nu voor de derde keer “afstuderen” en ik ben ontzettend dankbaar dat jullie mij die mogelijk hebben gegeven. Dit zal voorlopig de laatste keer zijn en ik ga nu eindelijk een “echte” baan zoeken, na een paar jaar Amerika dan.

Het promotieonderzoek beschreven in dit proefschrift, oorspronkelijk getiteld: “A combinatorial approach to probe the mechanism of binding of bivalent ligands to the GnRHR” is deel van het NWO-combichem programma, waar ik samen met Richard van den Berg aan begonnen ben; Ries, ik ben blij dat er iemand met mij in het bootje zat, zonder jou zou ik mij een stuk eenzamer hebben gevoeld. Het scheelt dat we nu nooit meer stofjes hoeven af te wegen in Oss!

Een aantal studenten zijn voor mij van grote betekenis geweest tijdens mijn promotieonderzoek. Marthe Walvoort, Annemiek Knijnenburg en Sascha Hoogendoorn; Dames, jullie zijn het bewijs dat vrouwen wel degelijk goede wetenschappers zijn! Jullie zijn nu alle drie terecht (bijna) AiO en ik wens jullie enorm veel succes in de toekomst. Er is versterking op komst!

Toen ik begon in de vakgroep Bio-organische Synthese, zijn mijn toenmalige - voornamelijk mannelijke - collega’s van grote invloed geweest op zowel mijn chemische, als persoonlijke ontwikkeling. Leendert van der Bos, Jeroen Codée, Dima Filippov, Gijsbert Grotenbreg, Farid El Oualid, Martijn de Koning, Bas Lastdrager, Michiel Leeuwenburgh, Remy Litjens en Mattie Timmer wil ik hier met name noemen. De meeste van jullie zijn vrij snel na mijn aanvang gepromoveerd of vertrokken maar ik heb in een korte tijd enorm veel van jullie geleerd!

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Ik wil in het bijzonder nog noemen Tom Wennekes; Tom, wij hebben samen het SAWU project uitgediept, en dat buiten ons eigen onderzoek. Ik heb de samenwerking altijd erg prettig gevonden en ik durf wel te stellen dat wij nu beide expert zijn in parallelle synthese. Hopelijk nooit kolom-blaren meer!

Ik heb tijdens dit onderzoek veel samengewerkt met de afdeling farmacochemie en in het bijzonder met Laura Heitman; Lau, je bent voor mij heel belangrijk geweest tijdens mijn onderzoek. Zonder jou had ik veel minder geleerd over receptoren en farmacologie. Ik weet nog goed dat ik mijn eerste celletje zag…

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Dit promotieonderzoek is in samenwerking met Schering-Plough research institute tot stand gekomen. Vele mensen van dit bedrijf zijn betrokken geweest in het managen, regelen en uitvoeren van verschillende analyses. Ten eerste wil ik Koen Thal en Saskia Verkaik bedanken; jullie zijn altijd zeel behulpzaam geweest in het regelen van de biologische testen en het in beginsel zuiveren van onze verbindingen op de preparatieve HPLC. Voor de betrokkenheid bij de biologische testen wil ik Maurice van Loosbroek, Monique van Amstel en Robert Tan zeer bedanken en in het bijzonder Julia Oosterom en Chris van Koppen; bedankt voor jullie tijd en inspanning om de niet altijd, of altijd niet eenvoudige biologische resultaten met mij te bespreken en te interpreteren. Paula Leandro Garcia wil ik bedanken voor het testen van de aggregatie eigenschappen van onze verbindingen en Jan Klomp en Marijn Sanders voor de zeer mooie model-platen van de dimere liganden met de GnRHR.
Buiten het lab spelen vrienden een enorm belangrijke rol; jullie begrip en interesse zijn voor mij van grote waarde geweest. Het is essentieel en erg fijn om naast het lab ontspanning te vinden in het “normale” leven.
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Stellingen

Behorende bij het proefschrift

Dimeric ligands for GPCRs involved in human reproduction: synthesis and biological evaluation

1. Het gebruik van de term bivalentie om het biologische effect van dimere liganden te beschrijven is dubbelzinnig.
   Dit proefschrift

2. De Lipinski regels vormen een belemmering voor de ontwikkeling van nieuwe medicijnen.
   Dit proefschrift
   Lipinski, C. A.; Lombardo, F.; Domini, B. W.; Feeney, P. J. *Adv. Drug. Deliv. Rev*. **2001**, *46*, 3-26.

3. Er komt bij heterocyclische chemie meer kijken dan enkel water uitstoken.
   Dit proefschrift

4. Samenwerking tussen universiteit en bedrijfsleven kan wel degelijk innovatief en vooruitstrevend onderzoek opleveren.
   Dit proefschrift
   http://www.sp.nl/zorg/columns/179/daarom_moeten_we_wetenschap_nationaliseren.html.

5. Het is de vraag of een groot aantal zelfcitaties zal leiden tot erkenning.
   Janovich, J. A.; Conn, P. M. *Endocrinology* **1996**, *137*, 3602-3605.

6. Het op verschillende manieren beschrijven van het biologisch profiel van eenzelfde verbinding leidt tot verwarring.
   Peng, X.; Knapp, B. I.; Bidlack, J. M.; Neumeyer, J. L. *J. Med. Chem*. **2007**, *50*, 2254-2258.

7. Het is de vraag of het anoniem indienen van artikelen zal leiden tot een eerlijkere beoordelingsprodedure van manuscripten geschreven door ‘ ster’-auteurs.
Bauch, H. *Nature* **2006**, *440*, 408.

8. Menig mannelijk intellect wordt niet bevorderd door de aanwezigheid van blonde vrouwen.
Bry, C.; Follenfant, A.; Meyer, T. *J. Exp. Soc. Psychol.* **2008**, *44*, 751-757.
The Sunday Times, 18 november 2007, You Silly Boys: Blondes make men act dumb.

9. Het is niet verwonderlijk dat toppers in de bètawetenschap vaak alfamannetjes zijn.

10. Het ontbreken van een eventuele strafmaat bij aanklachten tegen God geeft aan hoe serieus we deze beschuldigingen moeten nemen.
http://www.ad.nl/buitenland/article1671686.ece, 18 september 2007

Leiden, 19 december 2008 Kimberly Bonger