Chapter 2

Synthesis and evaluation of homodimeric GnRHR ligands

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 Gαq 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.4-9 Imidazopyrimidinone 17-9 (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. 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. 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) or with renilla luciferase and YFP.

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 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. 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 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 pKa 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. 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.

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\text{Scheme 1. Synthesis of the intermediate building block 12. Reagents and conditions: } \begin{align*}
\text{i. } & \text{NaOEt, EtOH, rt, 4 d, 79\%; ii. } \alpha\text{-bromopropiophenone, KI, K}_2\text{CO}_3, \text{DMF, rt, 5 d, 39\%; iii. } \text{Zn, HOAc, 80 °C, 6 h, 88\%; iv. } 2, 6\text{-difluorobenzyl bromide, KI, K}_2\text{CO}_3, \text{DMF, rt, 18 h, 70\%; v. } \text{NaNO}_3, \text{conc. } \text{H}_2\text{SO}_4, 0 °\text{C, 4 h, 82\%; vi. } \text{NBS, AIBN, CCl}_4, 80^°\text{C, 2 h, 83\%.}}
\end{align*}
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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 amine 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.

\[ \text{Scheme 2. Synthesis of acetylene ligands. Reagents and conditions: } \]

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i. \text{ } N \text{-methyl-} N \text{-benzylamine, DiPEA, THF, rt, 18 h, 97%}; ii. \text{ Fe, conc HCl, EtOH, rt, 4 h, 85%}; iii. \text{ 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. } \text{1M LiOH, H}_2\text{O/MeOH/THF (12/2/1; v/v/v), rt, 1 h, 83%}; v. \text{ propargylamine, BOP, DiPEA, DMF, rt, 18 h, 75%}; vi. \text{ tert-butyl 2-(benzylamino)acetate, DiPEA, THF, rt, 18 h, 97%}; vii. \text{ Fe, TFA/DCM (1/1; v/v), rt, 18 h, 97%}; viii. \text{ ethyl isocyanate, pyridine, rt, 18 h, 71%}; ix. \text{ 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. CuSO₄, sodium ascorbate, tBuOH/CH₃CN/H₂O (2/1/1; v/v/v), 60 °C, 3 h; ii. KOH, TsCl, DCM, 0 °C, 3 h, >95%; iii. NaN₃, 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 (EC80) 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 IC50 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 [125I]triptorelin. At first hand, single-point assay for all compounds was performed, in which the percentage of [125I]triptorelin displacement at 1 μg459M compound was determined (Column B). Furthermore, for a small number of ligands, including the reference compounds and a few dimeric ones, Ki 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. IC50 values (column A in Table 1) are always lower than the available corresponding Ki 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 (Ki) 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.
Table 1. Functional and binding properties to GnRHR for the mono- and dimeric ligands. The mean IC\textsubscript{50} are calculated from the –log IC\textsubscript{50} values from two or three independent experiments performed in duplicate. The SD of pIC\textsubscript{50} is generally lower than 0.3. \textsuperscript{a} CHO cells that stably express the GnRHR were stimulated with a submaximal (EC\textsubscript{80}) concentration of GnRH and were incubated with increasing concentrations of the compounds. The IC\textsubscript{50} value is the concentration of compound needed to inhibit the agonistic response by 50%. \textsuperscript{b} Membranes of GnRHR-expressing CHO cells were incubated with the radioactive GnRHR agonist [\textsuperscript{125}I]triptorelin. The percentage of [\textsuperscript{125}I]triptorelin displacement by incubating the fractions with a single concentration of 1 μM of test compound (Column B); K\textsubscript{i} values were determined (Column C) by incubating the membranes with increasing concentrations of test compound. The K\textsubscript{i} value is calculated based on the concentration of compound needed to displace 50% of radioligand. The mean K\textsubscript{i} are calculated from the -log K\textsubscript{i} values from three independent experiments performed in duplicate. The SD of pK\textsubscript{i} is generally lower than 0.2. [\textsuperscript{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 Geneticin. 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 (ECₘₐₓ) when given alone. After 4 h stimulation, 15 μL of lucite® 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). 

[^225]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% CO₂. 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 (31,000 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 MgCl₂, 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
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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 MgCl₂ 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 [¹²⁵I]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 MgCl₂ 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 (Ki values) were derived from the IC₅₀ values according to Ki = IC₅₀/(1 + [C]/Kd) where [C] is the concentration of the radioligand and Kd its dissociation constant. The Kd value (0.35 nM) of [¹²⁵I]Triptorelin was obtained by computer analysis of saturation curves. 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 (NH₄)₆Mo₇O₂₄ × 4H₂O (25 g/L) or (NH₄)₄Ce(SO₄)₂ × 2H₂O (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 ¹³C-APT spectra are proton decoupled. For LC-MS analysis, a HPLC-system (detection simultaneously at 214 and 254 nm) equipped with an analytical C₈ column (4.6 mmD × 250 mmL, 5μ particle size) in combination with buffers A: H₂O, B: CH₃CN and C: 1% aq. TFA and coupled to a mass instrument with an electrospray interface (ESI) was used. For RP-HPLC purifications, an automated HPLC system equipped with a preparative C₁₈ column (5 μm C₁₈, 10Å, 150 × 21.2 mm) was used. The applied buffers were A: H₂O + 0.1% TFA and B: CH₃CN. 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 = 400-2000) and dioctylphthalate (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]⁺. ¹H NMR (400 MHz, DMSO-d₆) δ 8.23 (s, 1H), 4.11 (q, 2H, J = 7.2), 1.21 (t, 3H, J = 6.8). ¹³C NMR (100 MHz, DMSO-d₆) δ 163.9 (C), 163.0 (CH), 158.7, 158.2, 103.6 (3 × C), 58.9 (CH₂), 13.8 (CH₃).
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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 CHCl₃ (500 mL) and washed with water (3 x 200 mL). The organic phase was dried (Na₂SO₄) 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 Rf = 0.7 (5% MeOH in DCM). ESI-MS m/z: 430.1 [M + H]+, 452.1 [M + Na]+. 1H NMR (200 MHz, CDCl₃) 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). 13C NMR (50 MHz, CDCl₃) 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 (CH₂), 57.0 (CH), 16.5, 14.3, 12.9 (3 × CH₃).

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 (CHCl₃/Et₂O/petroleum light ether) to yield white powder (3.05 g, 10.3 mmol, 88%). TLC Rf = 0.45 (10% MeOH in DCM). ESI-MS m/z: 297.9 [M + H]+, 595.2 [2M + H]+. 1H NMR (600 MHz, CDCl₃/CD3OD) 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). 13C NMR (150 MHz, CDCl₃/CD3OD) 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 (CH₂), 13.3, 10.8 (2 × CH₃).

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 (Na₂SO₄) and concentrated. The residue was crystallized from CHCl₃ and Et₂O to yield 6.08 g (14.3 mmol, 70%) of off-white crystals. TLC Rf = 0.75 (50% EtOAc in toluene). ESI-MS m/z: 424.0 [M + H]+, 847.5 [2M + H]+. 1H NMR (600 MHz, CDCl₃) δ 8.36 (s, 1H), 7.68 (d, 2H, J = 7.5), 7.43 (t, 2H, J = 7.5), 7.39 – 7.37 (m, 3H), 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). 13C NMR (150 MHz, CDCl₃) δ 164.2 (C), 161.7 (dd, 2 × C, J₁ = 250.0, J₂ = 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, J₁ = 21.0, J₂ = 4.5), 109.6 (t, 1 × C, J = 18.0), 100.9 (C), 61.11, 42.9 (2 × CH₃), 14.3, 12.21 (2 × CH₃).

8-(2, 6-Difluorobenzyl)-5, 8-dihydro-3-methyl-5-oxo-2-phenylimidazo[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 sodium nitrate (0.40 g, 4.74 mmol). After 4 h, the reaction mixture was poured on ice (100 mL) and CHCl₃ was added (150 mL). The organic layer was separated and the water layer extracted with EtOAc (2 × 200 mL) and the combined organic layers were dried (Na₂SO₄) and concentrated. The residue was crystallized from CHCl₃ and Et₂O to yield 1.83 g (3.91 mmol, 82%) of yellow crystals. TLC Rf = 0.75 (50% EtOAc in toluene). ESI-MS m/z: 469.2 [M + H]+. 1H NMR (400 MHz, CDCl₃) 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). 13C NMR (100 MHz, CDCl₃) δ 163.7 (C), 161.7 (dd, 2 × C, J₁ = 250.0, J₂ = 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 × CH₃), 14.3, 12.5 (2 × CH₃).
Dimeric GnRHR ligands

3-Bromomethyl-8-(2, 6-difluorobenzyl)-5, 8-dihydro-2-(4-nitrophenyl)-5-oxoimidazo[1, 2-alpyrimidine-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'-azobisisobutyronitrile (0.33 g, 2.0 mmol). The reaction mixture was stirred at 80 °C until a clear solution was observed (2-3 h). The volatiles were removed and the residue was dissolved in CHCl3 (200 mL). The organic layer was washed with aqueous NaHCO3 (200 mL) and the water layer was extracted with CHCl3 (3 x 100 mL). The combined organic layers were dried (Na2SO4) and concentrated. The residue was crystallized from DCM and Et2O to yield 4.55 g (83%) of brown crystals. TLC Rf = 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/CDCl3) 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).

13C NMR (500 MHz, MeOD/CDCl3) δ 163.1 (C), 161.4 (dd, 2 × C, J1 = 250.0, J2 = 6.3), 156.0 (C), 147.1, 141.6, 140.1, 138.4 (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 × CH2), 13.8 (CH3).

8-(2, 6-Difluorobenzyl)-5, 8-dihydro-3-(N-methyl-N-benzylaminomethyl)-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 CHCl3 (200 mL) and the organic layer washed with aqueous NaHCO3 (10%, 200 mL). The water layer was extracted with CHCl3 (3 x 100 mL) and the combined organic layers dried (Na2SO4) 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 Rf = 0.7 (50% EtOAc in toluene). ESI-MS m/z: 588.3 [M + H]+.

1H NMR (600 MHz, CDCl3) 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).

13C NMR (150 MHz, CDCl3) δ 164.2, 161.8 (dd, 2 × C, J1 = 257.8), 156.4, 146.6 (2 × C), 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, J1 = 250.5, J2 = 6.0), 109.5 (t, 1 × C, J = 18.0), 101.6 (C), 61.3 (2 × CH2), 49.7, 43.6 (2 × CH2), 41.6, 14.4 (2 × CH3).

2-(4-Aminophenyl)-8-(2, 6-difluorobenzyl)-5, 8-dihydro-3-(N-methyl-N-benzylaminomethyl)-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 NaHCO3 (10% 300 mL), CHCl3 (300 mL) and Hyflo were added. The mixture was filtered, the organic layer was separated and the water layer was extracted with CHCl3 (3 x 100 mL) and the combined organic layers dried (Na2SO4) 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 Rf = 0.7 (50% EtOAc in toluene). ESI-MS m/z: 588.3 [M + H]+.

1H NMR (200 MHz, CDCl3) 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). 13C NMR (200 MHz, CDCl3) δ 164.2, 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 × CH2), 41.1, 14.3 (2 × CH3).
8-(2, 6-Difluorobenzyl)-5, 8-dihydro-2-(4-ethylaminocarboxylaminophenyl)-3-(N-methyl-N-benzylaminomethyl)-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 NaHCO₃ (10%, 25 mL), dried (Na₂SO₄) 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

RF = 0.6 (10% MeOH in DCM). ESI-MS

m/z: 629.5 [M + H]+, 1257.8 [2M + H]2+. 1H NMR (500 MHz, CDCl₃) /g303 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), 7.42 – 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). 13C NMR (125 MHz, CDCl₃) /g303 163.2 (C), 161.5 (dd, 2 × C, J₁ = 250.0, J₂ = 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, J₁ = 20.0, J₂ = 3.8), 109.3 (t, 1 × C, J = 18.8), 100.4 (C), 60.4, 60.2, 49.7, 42.1 (4 × CH₂), 40.5 (CH₃), 33.8 (CH₂), 14.9, 13.7 (2 × CH₃). HRMS (m/z) calcd for C₃₄H₃₄N₆O₄F₂ + H+: 629.26824, obsd 629.26963.

8-(2, 6-Difluorobenzyl)-5, 8-dihydro-2-(4-ethylaminocarboxylaminophenyl)-3-(N-methyl-N-benzylaminomethyl)-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/H₂O) to yield an off-white solid (0.88 g, 1.5 mmol, 83%). TLC analysis (20% MeOH in DCM) RF = 0.10. ESI-MS

m/z: 601.4 [M + H]+, 1201.6 [2M + H]2+. 1H NMR (200 MHz, CDCl₃) /g303 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). 13C (100 MHz, DMSO-d₆) /g303 162.6 (C), 160.1 (dd, 2 × C, J₁ = 249.5, J₂ = 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 × CH₂), 110.0 (d, 2 × CH, J = 24.2), 108.6 (t, 1 × C, J = 18.8), 100.4 (C), 93.9, 48.4, 42.4 (3 × CH₂), 39.5 (CH₃), 32.4 (CH₂), 13.8 (CH₃).

8-(2, 6-Difluorobenzyl)-5, 8-dihydro-2-(4-ethylaminocarboxylaminophenyl)-3-(N-methyl-N-benzylaminomethyl)-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 NaHSO₃ (10%, 100 mL), NaHCO₃ (10%, 100 mL) and water (100 mL). The organic layer was dried (MgSO₄) 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-d₆) /g303 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). 13C NMR (125 MHz, DMSO-d₆) /g303 162.3 (C), 161.1 (dd, 2 × C, J₁ = 251.3, J₂ = 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 × CH₃), 41.0 (CH₃), 33.9, 28.2 (2 × CH₂), 15.4 (CH₃). HRMS m/z calcd for C₅₅H₄₅N₇O₄F₂ + H+: 638.26857, obsd 638.26710.
Dimeric GnRHR ligands

8-(2, 6-Difluorobenzyl)-5, 8-dihydro-2-(4-propargylaminocarboxylaminophenyl)-3-(N-methyl-N-benzylaminomethyl)-5-oximidazo[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 CHCl₃ (200 mL) and the mixture was washed with NaHCO₃ (3 × 100mL). The organic layer was dried (MgSO₄) 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]+. 1H NMR (400 MHz, DMSO-d₆) 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). 13C NMR (100 MHz, DMSO-d₆) δ 163.7 (C), 161.4 (dd, 2 × C, J₁ = 248.4, J₂ = 7.7), 155.9, 154.6 (2 × C), 147.9 (CH), 141.0, 139.9, 139.8, 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 (1 × CH, J = 18.2), 100.0 (C), 82.0 (CH), 72.7 (C), 60.7, 60.2, 49.6, 43.7 (4 × CH₂), 40.8 (CH₃), 28.7 (CH₂), 14.2 (CH₃). HRMS m/z calcd for C₃₅H₃₂N₆O₄F₂ + 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-oximidazo[1, 2-a]pyrimidine-6-carboxylic acid ethyl ester (16). To a cooled (0 ºC) 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 CHCl₃ (200 mL) and the organic layer washed with aqueous NaHCO₃ (10%, 200 mL). The water layer was extracted with CHCl₃ (3 × 100 mL) and the combined organic layers were dried (Na₂SO₄) 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 Rf = 0.8 (50% EtOAc in toluene). ESI-MS m/z: 688.4 [M + H]+, 710.6 [M + Na]+. 1H NMR (400 MHz, CDCl₃) 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). 13C NMR (400 MHz, CDCl₃) δ 170.3, 163.1 (2 × C), 161.4 (dd, 2 × C, J₁ = 250.0, J₂ = 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 (1 × C, J = 18.0), 101.2, 80.0 (2 × C), 60.8, 57.9, 55.0, 47.2, 43.3 (5 × CH₂), 27.7, 14.0(4 × CH₃). HRMS m/z calcd for C₃₆H₃₅F₂N₅O₇ + H+: 688.25773, obsd 688.25557.

2-((N-(6-(Ethoxycarbonyl)-8-(2, 6-difluorobenzyl)-2-(4-aminophenyl)-5, 8-dihydro-5-oximidazo[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 ºC) 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 NaHCO₃ (10%, 300 mL) and Celite were added. The mixture was filtered, the organic layer dispersed and the water layer extracted with CHCl₃ (3 × 100 mL) and Celite were added. The mixture was filtered, the organic layer dispersed and the water layer extracted with CHCl₃ (3 × 200 mL). The combined organic layers were dried (Na₂SO₄) and concentrated. Column chromatography of the residue (0% to 50% MeOH in EtOAc to toluene) afforded 3.40 g (4.9 mmol, 97%) as an off-white solid. TLC Rf = 0.8 (50% EtOAc in toluene). ESI-MS m/z: 625.5 [M + H]+, 623.3 [2M + H]+. 1H NMR (400 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 (q, 2H, J = 7.3), 4.10 (s, 2H), 1.38 (t, 3H, J = 7.3). 13C NMR (50 MHz, MeOD/TFA) δ 170.0 (C), 163.0 (d, 2 × C, J = 25.00), 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 = 22.7), 111.2 (t, 1 × C, J = 18.2), 101.9 (C), 62.1, 60.0, 56.9, 50.7, 44.8 (5 × CH2), 41.6 (CH3).

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 NaHCO3 (10%, 100 mL), dried (Na2SO4) 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]+. 1H NMR (400 MHz, CDCl3/TFA) /g303 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). 13C NMR (400 MHz, CDCl3/TFA) /g303 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 × CH2), 14.6, 13.8 (2 × CH3). HRMS m/z calcd for C35H34N6O6F2 + H+: 673.25807, obsd 673.25730.

8-(2, 6-Difluorobenzyl)-5, 8-dihydro-2-(4-ethylaminocarbonylaminophenyl)-3-(N-propargyl amidomethyl-N-benzylaminomethyl)-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, 1.95 mmol) 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 NaHSO3 (10%, 100ml), NaHCO3 (10%, 100ml) and water (100ml). The organic layer was dried (MgSO4) 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/CHCl3 and Et2O 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]+. 1H NMR (500 MHz, DMSO-d6/TFA) /g303 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). 13C NMR (500 MHz, DMSO/TFA) δ 163.9, 163.0 (2 × C), 161.2 (dd, 2 × C, J = 251.0, J = 7.2), 157.9, 154.3 (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 × CH2), 15.5, 14.3 (2 × CH3). HRMS m/z calcd for C39H39N7O6F2 + 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 (MgSO4) 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). ¹³C NMR (50 MHz, CDCl₃) δ 144.9, 132.7 (4 × C), 129.8, 127.8 (8 × CH), 69.0, 68.6 (4 × CH₂), 21.5 (2 × CH₃).

1, 5-Ditosyl-3, 6-dioxapentane (20c). ¹H NMR (200 MHz, CDCl₃) 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). ¹³C NMR (50 MHz, CDCl₃) δ 144.7, 132.7 (4 × C), 129.8, 127.8 (8 × CH), 70.5, 69.1, 68.4 (6 × CH₂), 21.5 (2 × CH₃).

1, 8-Ditosyl-3, 6-dioxaoctane (20d). ¹H NMR (200 MHz, CDCl₃) 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). ¹³C NMR (50 MHz, CDCl₃) δ 144.7, 132.7 (4 × C), 129.7, 127.8 (8 × CH), 70.5, 70.3, 69.1, 68.4 (10 × CH₂), 21.5 (2 × CH₃).

1, 11-Ditosyl-3, 6, 9-trioxaundecane (20e). ¹H NMR (200 MHz, CDCl₃) 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). ¹³C NMR (50 MHz, CDCl₃) δ 144.7, 132.7 (4 × C), 129.7, 127.8 (8 × CH), 70.6 70.5, 70.3, 69.1, 68.4 (10 × CH₂), 21.5 (2 × CH₃).

1, 14-Ditosyl-3, 6, 9, 12-tetraoxatetradecane (20f). ¹H NMR (200 MHz, CDCl₃) δ 7.78 (d, 4H, J = 8.0), 7.34 (d, 4H, J = 8.4), 4.17 - 4.13 (m, 16H), 3.67 - 3.62 (m, 4H), 3.55 (s, 4H), 2.44 (s, 6H). ¹³C NMR (50 MHz, CDCl₃) δ 144.7, 132.7 (4 × C), 129.7, 127.8 (8 × CH), 70.5 70.4, 70.3, 69.1, 68.4 (10 × CH₂), 21.5 (2 × CH₃).

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). NaN₃ (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. Et₂O was added and the resulting solids were filtered. The Et₂O was evaporated and the concentration of the final solution of 1, 2-diazidoethane in DMF was determined by ¹H NMR and was used without further purification. ¹H NMR (500 MHz, CDCl₃) δ 3.53 (s, 4H). ¹³C NMR (125 MHz, CDCl₃) δ 49.0 (2 × CH₂). ATR-IR (thin film): 2100 (N₃) cm⁻¹.

General procedure for the synthesis of bis-azide ethyleneglycols 21. Ditosylated ethyleneglycol 20 (150 mmol) was dissolved in DMF (225 mL). NaN₃ (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 Et₂O. 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). ¹H NMR (200 MHz, CDCl₃) δ 3.67 (t, 4H, J = 4.7), 3.40 (t, 4H, J = 5.1). ¹³C NMR (50 MHz, CDCl₃) δ 69.4, 50.4 (4 × CH₂). ATR-IR (thin film): 2089 (N₃), 1124 (C-O) cm⁻¹.

1, 8-Diazido-3, 6-dioxaoctane (21c). ¹H NMR (200 MHz, CDCl₃) δ 3.64 - 3.56 (m, 8H), 3.31 (t, 4H, J = 5.1). ¹³C NMR (50 MHz, CDCl₃) δ 70.4, 69.8, 50.4 (6 × CH₂). ATR-IR (thin film): 2095 (N₃), 1115 (C-O) cm⁻¹.

1, 11-Diazido-3, 6, 9-trioxaundecane (21d). ¹H NMR (200 MHz, CDCl₃) δ 3.56 - 3.54 (m, 12H), 3.27 (t, 4H, J = 5.1). ¹³C NMR (50 MHz, CDCl₃) δ 70.3, 69.6, 50.3 (8 × CH₂). ATR-IR (thin film): 2091 (N₃), 1090 (C-O) cm⁻¹.

1, 14-Diazido-3, 6, 9, 12-tetraoxatetradecane (21e). ¹H NMR (200 MHz, CDCl₃) δ 3.62 - 3.60 (m, 16H), 3.34 (t, 4H, J = 5.1). ¹³C NMR (50 MHz, CDCl₃) δ 70.5, 69.9, 50.5 (10 × CH₂). ATR-IR (thin film): 2095 (N₃), 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 tBuOH/CH3CN/H2O (2/1/1; v/v/v, 900 μL) were added sodium ascorbate (1 eq. 50 μL of a 1M solution in H2O) and CuSO4 (0.2 eq. 50 μL of a 0.2M solution in H2O). 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/H2O/CH3CN/TFA (10/1/1/1; 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: tR 6.94 min (linear gradient 10 to 90% B in 13.5 min; m/z: 750.4 [M + H]+). 1H NMR (500 MHz, DMSO-d6) 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 x 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 C39H41N13O4F2 + H+: 794.3459, obsd 794.34299.

**Monomeric ligand 22b.** Yield after RP-HPLC purification: 16.1 mg (17.7 μmol, 35%). LC-MS analysis: tR 6.82 min (linear gradient 10 to 90% B in 13.5 min; m/z: 794.5 [M + H]+). 1H NMR (500 MHz, DMSO-d6) 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, J1 = 14.5, J2 = 8.7), 7.50 – 7.42 (m, 6H), 7.16 (t, 2H, J = 8.1), 6.93 (br s, 1H), 5.70 (s, 2H), 5.15 – 5.11 (m, 1H), 4.81 – 4.76 (m, 1H), 4.63 (dd, 2H, J1 = 32.9, J2 = 15.2, J3 = 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 C30H36N13O6F2 + H+: 794.34453, obsd 794.34299.

**Monomeric ligand 22c.** Yield after RP-HPLC purification: 22.3 mg (23.4 μmol, 47%). LC-MS analysis: tR 7.25 min (linear gradient 10 to 90% B in 13.5 min; m/z: 838.4 [M + H]+). 1H NMR (500 MHz, DMSO-d6) 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, J1 = 20.9, J2 = 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 C31H38N13O6F2 + H+: 838.37074, obsd 838.36890.

**Monomeric ligand 22d.** Yield after RP-HPLC purification: 15.8 mg (15.8 μmol, 32%). LC-MS analysis: tR 5.75 min (linear gradient 10 to 90% B in 13.5 min; m/z: 882.4 [M + H]+). 1H NMR (500 MHz, DMSO-d6) 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, J1 = 21.8, J2 = 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, J1 = 15.1, J2 = 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 C40H40N13O6F2 + H+: 882.39696, obsd 882.39498.

**Monomeric ligand 22e.** Yield after RP-HPLC purification: 18.6 mg (17.9 μmol, 36%). LC-MS analysis: tR 7.24 min (linear gradient 10 to 90% B in 13.5 min; m/z: 926.2 [M + H]+). 1H NMR (500 MHz, DMSO-d6) 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, J1 = 17.8, J2 = 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, J1 = 8.7, J2 = 5.2), 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 C41H40N13O6F2 + H+: 926.4 [M + H]+.
Monomeric ligand 23a. Yield after RP-HPLC purification: 6.9 mg (8.0 µmol, 16%). LC-MS analysis: tR 7.21 min (linear gradient 10 to 90% B in 13.5 min; m/z: 751.4 [M + H]+). 1H NMR (500 MHz, DMSO-d6) 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_{39}H_{40}N_{12}O_{5}F_{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: tR 7.34 min (linear gradient 10 to 90% B in 13.5 min; m/z: 795.3 [M + H]+). 1H NMR (500 MHz, DMSO-d6) 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), 5.05 (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_{5}F_{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: tR 7.38 min (linear gradient 10 to 90% B in 13.5 min; m/z: 839.3 [M + H]+). 1H NMR (500 MHz, DMSO-d6) 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 = 5.6), 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_{45}H_{46}N_{12}O_{6}F_{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: tR 7.59 min (linear gradient 10 to 90% B in 13.5 min; m/z: 883.3 [M + H]+). 1H NMR (500 MHz, DMSO-d6) 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_{45}H_{45}N_{12}O_{6}F_{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: tR 7.38 min (linear gradient 10 to 90% B in 13.5 min; m/z: 927.5 [M + H]+). 1H NMR (500 MHz, DMSO-d6) 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_{46}N_{12}O_{6}F_{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: tR 7.47 min (linear gradient 10 to 90% B in 13.5 min; m/z: 882.4 [M + H]+). 1H NMR (500 MHz, DMSO-d6) δ 8.96 (s, 1H), 8.79 (br s, 2H), 7.90 (s, 1H), 7.51 (dd, 4H, J = 13.8, J = 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_{45}H_{46}N_{12}O_{6}F_{2} + H+: 882.33944, obsd 882.33927.
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-d6) 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), 3.16 – 3.08 (m, 2H), 1.33 (t, 3H, *J* = 7.2). HRMS *m/z* caled for C42H45N13O6F2 + 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-d6) 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), 3.58 – 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* caled for C44H49N13O7F2 + 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-d6) 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* = 5.2), 3.58 – 3.49 (m, 6H), 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-d6) 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), 4.00 – 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* caled for C48H57N13O9F2 + 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/H2O (2/1/1; v/v/v, 800 μL) were added sodium ascorbate (1 eq. 100 μL of a 1M solution in H2O) and CuSO4 (0.2 eq. 100 μL of a 0.2M solution in H2O). 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/H2O/CH3CN/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-d6) δ 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
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]^+). 1H NMR (500 MHz, DMSO-d6) 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 calec for C_{32}H_{36}N_{10}O_{4}F_4+: 1387.57961, obsd 1387.59127.

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]^+). 1H NMR (500 MHz, DMSO-d6) δ 9.12 (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 calec for C_{30}H_{28}N_{10}O_{4}F_4+: 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]^+). 1H NMR (500 MHz, DMSO-d6) 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 calec for C_{36}H_{32}N_{10}O_{4}F_4+: 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]^+). 1H NMR (500 MHz, DMSO-d6) 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 calec for C_{36}H_{32}N_{10}O_{4}F_4+: 1563.68447, obsd 1563.68651.

Dimeric ligand 26a. Yield after RP-HPLC purification: 27.9 mg (17.3 µmol, 35%). LC-MS analysis: t_R 7.50 min (linear gradient 10 to 90% B in 13.5 min; m/z: 1389.5 [M + H]^+). 1H NMR (500 MHz, DMSO-d6) 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 calec for C_{29}H_{38}N_{10}O_{4}F_4+: 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]^+). 1H NMR (500 MHz, DMSO-d6) 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 calec for C_{29}H_{38}N_{10}O_{4}F_4+: 1433.57385, obsd 1433.58036.
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**Dimeric ligand 26c.** Yield after RP-HPLC purification: 25.6 mg (15.0 μmol, 30%). LC-MS analysis: 

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**Dimeric ligand 26d.** Yield after RP-HPLC purification: 26.0 mg (14.9 μmol, 30%). LC-MS analysis: 

**Dimeric ligand 26c.** Yield after RP-HPLC purification: 31.1 mg (17.3 μmol, 35%). LC-MS analysis: 

**Dimeric ligand 26d.** Yield after RP-HPLC purification: 31.1 mg (17.3 μmol, 35%). LC-MS analysis: 

**Dimeric ligand 27a.** Yield after RP-HPLC purification: 15.3 mg (8.7 μmol, 17%). LC-MS analysis: 

**Dimeric ligand 27b.** Yield after RP-HPLC purification: 41.9 mg (23.2 μmol, 46%). LC-MS analysis: 

**Dimeric ligand 27c.** Yield after RP-HPLC purification: 10.8 mg (7.0 μmol, 14.0%). LC-MS analysis: 

**Dimeric ligand 27d.** Yield after RP-HPLC purification: 32.1 mg (17.0 μmol, 34.0%). LC-MS analysis: 

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**Dimeric ligand 27e.** Yield after RP-HPLC purification: 20.0 mg (10.3 μmol, 21%). LC-MS analysis: *t* <sub>R</sub> 7.85 min (linear gradient 10 to 90% B in 13.5 min; *m/z*: 1707.6 [M + H]+). ¹H NMR (500 MHz, DMSO-<em>d</em>₆) δ 8.96 (s, 2H), 8.83 (br s, 4H), 7.51 – 7.07 (m, 24H), 6.20 (br s, 2H), 5.56 (s, 4H, CH<sub>2</sub>C₆H₄F₂), 5.10 – 4.65 (br s, 4H), 4.55 – 4.43 (br s, 4H), 4.38 (t, *J* = 4.5), 4.22 (q, *J* = 6.9), 4.02 (br s, 4H), 3.88 (br s, 4H), 3.69 (t, *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 C₈₆H₉₄N₂₀O₁₄F₄ + H+: 1707.72673, obsd 1707.74903.

**References and notes**

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