Allosteric Modulation of 'Reproductive' GPCRs : a case for the GnRH and LH receptors

Heitman, L.H.

Citation

Heitman, L. H. (2009, April 22). *Allosteric Modulation of 'Reproductive' GPCRs : a case for the GnRH and LH receptors*. Retrieved from https://hdl.handle.net/1887/13748

Version: Corrected Publisher's Version

License: Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden

Downloaded from: https://hdl.handle.net/1887/13748

Note: To cite this publication please use the final published version (if applicable).

CHAPTER 5

SUBSTITUTED TERPHENYL COMPOUNDS AS THE FIRST CLASS OF LOW MOLECULAR WEIGHT ALLOSTERIC INHIBITORS OF THE LUTEINIZING HORMONE RECEPTOR

The luteinizing hormone (LH) receptor plays an important role in fertility and certain cancers. The endogenous ligands, human chorionic gonadotropin (hCG) and LH bind to the large N terminal domain of the receptor. We recently reported on the first radiolabeled low molecular weight (LMW) agonist for this receptor, [^{3}H]Org 43553, which was now used to screen for new LMW ligands. We identified a terphenyl derivative that inhibited [^{3}H]Org 43553 binding to the receptor, which led us to synthesize a number of derivatives. The most potent compound of this terphenyl series **24** (LUF5771) was able to increase the dissociation rate of [^{3}H]Org 43553 by 3.3-fold (at 10 µM). In a functional assay, the presence of **24** resulted in a 2- to 3-fold lower potency of both Org 43553 and LH. Thus, the compounds presented in this paper are the first LMW ligands that allosterically inhibit the LH receptor.

5.1 INTRODUCTION

The luteinizing hormone (LH) receptor is a member of the glycoprotein hormone receptor family within the class A subfamily of G protein-coupled receptors (GPCRs).[18] While most class A GPCRs recognize low molecular weight (LMW) endogenous ligands that bind in the seven transmembrane (7-TM) domain, the LH receptor has two high molecular weight endogenous ligands, human chorionic gonadotropin (hCG) and LH. Both hormones bind with high affinity and selectivity to the N terminus of the LH receptor and thereby activate the receptor.[259] In the clinic, these so-called gonadotropins are currently used in infertility treatment. The hormones need to be administered by parenteral (subcutaneous or intramuscular) injection.[187]

To increase patient convenience and compliance, efforts are made to develop non-peptide orally active gonadotropins as drugs. For the LH receptor only a few compound classes have been described as LMW receptor agonists, such as thienopyrimidine, Org 43553. This compound was shown to have *in vivo* efficacy upon oral administration.[69] Recently the first high molecular weight antagonist was reported for the LH receptor, two fused beta-subunits of hCG.[189] However, LMW antagonists have not been reported so far (*Chapter 2*). Antagonists for the LH receptor may be novel contraceptive agents. In addition, antagonists could be used against ovarian cancer related to menopause.[80] Therefore, next to LMW agonists, antagonists would be very beneficial as well.

Recently, we reported on the first radiolabeled LMW agonist for the LH receptor, [^{3}H]Org 43553 (described in *Chapter 4*). Here, we used this radioligand to screen for new LMW ligands at the LH receptor. Initially, 50 compounds were screened for displacement of [^{3}H]Org 43553. Subsequently, the same library was screened in a kinetic radioligand binding assay, where a change in dissociation rate is indicative for allosteric modulation of the radioligand used.[277] The latter resulted in a few hits including the terphenyl compound **4**, which we anticipated to be an allosteric inhibitor as it increased the dissociation rate of [^{3}H]Org 43553. Subsequently, several analogues of **4** were synthesized and tested for their effect on the dissociation rate of [^{3}H]Org 43553. This yielded an even more potent allosteric inhibitor, compound **24** (LUF5771) that was further characterized in radioligand dissociation experiments and functional assays. As a consequence, the present study is the first to report LMW allosteric inhibitors of the LH receptor.

5.2 MATERIALS AND METHODS

5.2.1 Chemistry - Materials

All reagents used were obtained from commercial sources and all solvents were of analytical grade. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 400 (^{1}H NMR, 400 MHz; ^{13}C NMR, 100 MHz) and Bruker AC 200 (^{1}H NMR, 200 MHz; ^{13}C NMR, 50 MHz) spectrometer with tetramethylsilane as an internal standard. Chemical shifts are reported in δ (ppm). Melting points were determined by Büchi melting point apparatus and are uncorrected. Elemental analyses were performed by Leiden Institute of Chemistry and are within 0.4% of theoretical values unless otherwise stated. Reactions were routinely monitored by TLC using Merck silica gel F254 plates. Microwave reactions were performed on an Emrys Optimizer (Biotage AB). Wattage was automatically adjusted to maintain the desired temperature.

5.2.2 General Methods for Microwave Assisted Suzuki–Miyaura Cross Coupling Reaction (6-10)

To a solution of 3,5-dibromophenol (1 eq) in toluene, $(Ph_3P)_4Pd$ (5 mol.%), substituted phenylboronic acid (3 eq), 2 M aq. Na_2CO_3 (2 eq) was added and the resulting mixture was heated in microwave at 150 ^{o}C for 10 min. Upon completion (TLC), the reaction mixture was diluted with EtOAc (100 mL), washed with H_2O, brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude compound was purified by flash column chromatography (EtOAc/petroleum ether, 1:9) to afford the desired product.

[1,1’,3’,1’’]Terphenyl-5’-ol (6). Low melting colorless solid, yield 71%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.69–7.58 (m, 5H), 7.44–7.33 (m, 5H), 7.03 (d, J = 1.6 Hz, 2H), 5.29 (brs, 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 156.3, 143.5, 140.8, 129.4, 128.9, 128.0, 127.7, 127.3, 117.4, 113.0 ppm.

4,4’’-Dichloro-1,1’,3’,1’’-terphenyl-5’-ol (7). Yield: 130 mg, 34%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.56 (d, J = 8.1 Hz, 4H), 7.41 (d, J = 8.4 Hz, 4H), 7.23 (s, 1H), 7.03 (s, 2H), 4.65 (brs, 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 157.7, 141.8, 139.4, 133.3, 128.6, 128.2, 117.0, 112.0 ppm.

3,3'',4,4''-Tetrachloro-1,1',3',1''-terphenyl-5'-ol (8). Yield: 280 mg, 61%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.78 (s, 2H), 7.53–7.48 (m, 4H), 7.25 (s, 1H), 7.03 (s, 2H), 4.63 (brs, 1H) ppm.

4,4''-Dimethoxy-1,1',3',1''-terphenyl-5'-ol (9). Yield: 150 mg, 41%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.51 (d, *J* = 8.8 Hz, 4H), 7.29 (s, 1H), 6.98–6.93 (m, 6H), 5.35 (brs, 1H) 3.82 (m, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 159.5, 156.4, 143.2, 133.6, 128.5, 118.3, 114.5, 111.4, 55.7 ppm.

4,4''-Dimethyl-1,1',3',1''-terphenyl-5'-ol (10). Yield: 220 mg, 67%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.51 (d, *J* = 8.0 Hz, 4H), 7.27 (s, 1H), 7.24 (d, *J* = 8.0 Hz, 4H), 6.99 (s, 2H), 4.90 (brs, 1H), 2.38 (s, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 145.7, 141.0, 139.6, 131.8, 129.2, 129.1, 119.3, 114.8 22.5 ppm.

5.2.3 Synthesis of 5-Bromo-biphenyl-3-ol (11)

To a stirred solution of 3,5-dibromophenol (11.95 mmol, 3 g) in toluene (50 mL), phenylboronic acid (12.55 mmol, 1.53 g), $(Ph_3P)_4Pd$ (0.6 mmol, 0.69 g), and 2 M aqueous solution of Na_2CO_3 (23.91 mmol, 2.53 g) was added and refluxed for 3 h. The progress of the reaction was monitored by TLC. Upon completion, the reaction was cooled to ambient temperature, diluted with EtOAc (150 mL), washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude compound was purified by column chromatography using 10% EtOAc in petroleum ether to obtain compound **11** as colorless oil (Yield: 0.89 g, 30%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.52 (d, *J* = 7.2 Hz, 2H), 7.43 (t, *J* = 7.2 Hz, 2H), 7.37 (d, *J* = 7.2 Hz, 1H), 7.31 (s, 1H), 6.98 (d, *J* = 2.8 Hz, 2H), 5.06 (brs, 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 154.5, 141.2, 135.1, 129.2, 128.4, 127.4, 125.1, 123.3, 117.8, 113.5 ppm.

5.2.4 General Methods for Microwave Assisted Suzuki–Miyaura Cross Coupling Reaction (12-15)

To a solution of 5-bromo-biphenyl-3-ol (**11**) in toluene, $(Ph_3P)_4Pd$ (5 mol.%), substituted phenylboronic acid (1.5 eq) and 2 M aq. Na_2CO_3 (2 eq) were added and the resulting mixture was heated in microwave at 150 °C for 10 min. Upon completion (TLC), the reaction mixture was diluted with EtOAc (100 mL), washed with H_2O, brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude compound was purified by flash column chromatography (EtOAc/petroleum ether, 1:9) to yield the desired product.

R2
OH
R1

4-Chloro-[1,1',3',1'']terphenyl-5'-ol (12). Yield: 200 mg, 67%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.65 (d, J = 8.0 Hz, 2H), 7.58 (d, J = 7.6 Hz, 2H), 7.49–7.36 (m, 5H), 7.34 (s, 1H), 7.03 (s, 1H), 6.98 (s, 1H), 5.14 (brs, 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 156.5, 143.9, 142.4, 140.8, 139.4, 129.3, 129.2, 129.1, 128.7, 128.0, 127.5, 119.1, 113.7, 113.2 ppm.

3,4-Dichloro-[1,1',3',1'']terphenyl-5'-ol (13). Yield: 265 mg, 70%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.69 (d, J = 2.0 Hz, 2H), 7.53–7.36 (m, 6H), 7.25 (s, 1H), 7.07 (s, 1H), 6.98 (s, 1H), 5.04 (brs, 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 155.5, 143.3, 141.1, 140.2, 139.1, 134.3, 132.2, 131.8, 129.8, 129.4, 128.5, 127.5, 126.7, 119.0, 114.2, 113.9 ppm.

4-Methoxy-[1,1',3',1'']terphenyl-5'-ol (14). Yield: 300 mg, 64%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.59 (d, J = 7.6 Hz, 2H), 7.53 (d, J = 8.4 Hz, 2H), 7.42 (t, J = 7.4 Hz, 2H), 7.36–7.33 (m, 2H), 6.99 (s, 2H), 6.96 (d, J = 8.4 Hz, 2H), 5.27 (brs, 1H), 3.83 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 159.6, 156.5, 143.7, 143.3, 141.2, 133.6, 129.1, 128.5, 127.9, 127.5, 118.8, 114.6, 113.0, 112.8, 55.7 ppm.

4-Methyl-[1,1',3',1'']terphenyl-5'-ol (15). Yield: 194 mg, 62%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.60 (d, J = 7.6 Hz, 2H), 7.51 (d, J = 8.0 Hz, 2H), 7.44 (t, J = 7.8 Hz, 2H), 7.38–7.34 (m, 2H), 7.25 (d, J = 7.2 Hz, 2H), 7.01 (s, 2H), 4.99 (brs, 1H), 2.42 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 155.6, 141.7, 140.3, 139.7, 139.1, 133.6, 129.8, 129.7, 129.1, 127.9, 127.5, 127.3, 119.1, 113.2, 113.1, 21.4 ppm.

5.2.5 General Procedure for Carbamate Synthesis (4, 16-25, 34-41)

Triethylamine (1.5 eq) was added to a stirred solution of phenol (1 eq) in anhydrous dichloromethane and stirred at ambient temperature for 15 min. Isocyanate (1.5 eq) was added and stirred for another 12 to 18 h at ambient temperature. Upon completion (TLC), the reaction mixture was diluted with dichloromethane (100 mL), washed with water, brine, dried and evaporated under reduced pressure. The crude compound was purified by flash column chromatography (dichloromethane/petroleum ether, 4:1) to get the carbamate. The column purified carbamate was crystallized using dichloromethane and petroleum ether.

Phenyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (4). Yield: 259 mg, 87%. M.p.: 165–166 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.68–7.61 (m, 5H), 7.51–7.31 (m, 12H), 7.17–7.05 (m, 2H), 4.08 (brs, 1H), 3.85 (s, 3H) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 164.1, 151.1, 143.0, 137.2, 129.1, 128.7, 127.6, 127.2, 123.9, 123.3, 119.1, 118.8 ppm. MS (ES$^+$): 366 (MH$^+$). Anal. ($C_{25}H_{19}NO_2$) C, H, N.

(4-Chloro-phenyl)-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (16). Yield: 290 mg, 89%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.67 (t, J = 1.6 Hz, 1H), 7.63–7.57 (m, 4H), 7.48–7.35 (m, 10H), 6.88 (td, J = 8.8 Hz, J = 2.4 Hz, 2H), 7.04 (brs, 1H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 151.5, 143.6, 140.5, 129.5, 129.2, 128.1, 127.6, 123.9, 119.5, 114.3 ppm. Anal. ($C_{25}H_{18}ClNO_2$) C, H, N.

(4-Methoxy-phenyl)-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (17). Yield: 300 mg, 93%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.67–7.59 (m, 5H), 7.47–7.35 (m, 10H), 6.88 (d, J = 8.8 Hz, 2H), 4.08 (brs, 1H), 3.85 (s, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 151.6, 143.1, 140.3, 128.8, 127.8, 127.2, 123.1, 120.8, 119.3, 114.3, 55.5 ppm. Anal. ($C_{26}H_{21}NO_3$) C, H, N.

Ethyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (18). Yield: 240 mg, 93%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.62–7.55 (m, 5H), 7.48–7.40 (m, 4H), 7.39–7.32 (m, 4H), 5.08 (brs, 1H), 3.38–3.29 (m, 2H), 1.24 (t, J = 7.2 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 152.1, 143.2, 140.7, 129.1, 129.0, 127.9, 127.8, 127.6, 127.5, 123.3, 119.6, 36.5, 15.4 ppm. Anal. ($C_{21}H_{19}NO_2 \cdot 0.5\ H_2O$) C, H, N.

Propyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (19). Yield: 180 mg, 66%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.65–7.60 (m, 5H), 7.46–7.41 (m, 4H), 7.38–7.32 (m, 4H),

5.12 (brs, 1H), 3.27–3.20 (m, 2H), 1.64–1.55 (m, 2H), 0.95 (t, J = 8.3 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 152.1, 143.2, 140.7, 129.1, 128.0, 127.6, 127.5, 123.3, 119.6, 43.3, 23.4, 11.6 ppm. Anal. ($C_{22}H_{21}NO_2 \cdot 0.5\ H_2O$) C, H, N.

Butyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (20). Yield: 240 mg, 85%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.63–7.60 (m, 5H), 7.46–7.39 (m, 4H), 7.38–7.33 (m, 4H), 5.08 (brs, 1H), 3.31–3.24 (m, 2H), 1.60–1.53 (m, 2H), 1.44–1.35 (m, 2H), 0.95 (t, J = 7.2 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 154.6, 143.0, 140.4, 129.5, 128.8, 127.7, 127.3, 127.2, 124.0, 123.0, 119.2, 41.0, 31.9, 19.9, 13.7 ppm. Anal. ($C_{23}H_{23}NO_2 \cdot 0.5\ H_2O$) C, H, N.

Pentyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (21). Yield: 130 mg, 89%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.66–7.61 (m, 5H), 7.45–7.40 (m, 4H), 7.38–7.31 (m, 4H), 5.10 (brs, 1H), 3.30–3.21 (m, 2H), 1.63–1.52 (m, 2H), 1.41–1.28 (m, 4H), 0.91 (t, J = 6.4 Hz, 3H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 152.0, 143.2, 140.7, 129.0, 128.1, 127.9, 127.5, 127.2, 123.2, 123.0, 119.5, 41.6, 31.9, 29.8, 22.6, 14.2 ppm. Anal. ($C_{24}H_{25}NO_2 \cdot 0.5\ H_2O$) C, H, N.

Isopropyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (22). Yield: 174 mg, 65%. M.p.: 160 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.67–7.64 (m, 4H), 7.50–7.36 (m, 9H), 7.39–7.32 (m, 4H), 4.97 (m, 1H), 4.00–3.90 (m, 1H), 1.27 (d, J = 6.6 Hz, 6H) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 151.6, 142.8, 140.3, 130.0, 128.7, 127.6, 127.2, 122.9, 119.2, 98.0, 43.4, 22.8 ppm. MS (ES^+): 332 (MH^+). Anal. ($C_{22}H_{21}NO_2$) C, H, N.

tert-Butyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (23). Yield: 200 mg, 71%. M.p.: 139 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.66–7.63 (m, 5H), 7.49–7.35 (m, 8H), 5.06 (m, 1H), 1.43 (s, 9H) ppm. ^{13}C NMR (50 MHz, $CDCl_3$): δ = 151.5, 142.8, 140.4, 128.7, 127.5, 127.2, 122.8, 119.3, 28.7 ppm. Anal. ($C_{23}H_{21}NO_2 \cdot 0.1\ H_2O$) C, H, N.

Cyclopentyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (24). Yield: 197 mg, 68%. M.p.: 136 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.67–7.64 (m, 4H), 7.51–7.38 (m, 9H), 5.18–5.14 (m, 1H), 4.16–4.10 (m, 1H), 2.07–2.04 (m, 2H), 1.70-1.30 (m, 6H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 153.9, 151.7, 142.8, 140.3, 128.7, 127.6, 127.2, 122.9, 119.2, 53.0, 33.1, 23.9 ppm. Anal. ($C_{24}H_{23}NO_2$) C, H, N.

Cyclohexyl-carbamic acid [1,1',3',1'']terphenyl-5'-yl ester (25). Yield: 139 mg, 70%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.74–7.64 (m, 5H), 7.54–7.47 (m, 4H), 7.45–7.39 (m, 4H), 5.13 (t, J = 7.6 Hz, 1H), 3.74–3.60 (m, 1H), 2.17–2.02 (m, 2H), 1.85–1.53 (m, 3H), 1.48–1.20 (m, 5H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 154.0, 152.1, 143.2, 140.7, 129.7,

129.0, 128.2, 127.9, 127.5, 123.1, 119.6, 50.5, 33.5, 25.7, 25.0 ppm. Anal. ($C_{25}H_{25}NO_2 \cdot 0.5 H_2O$) C, H, N.

4-Chloro-1,1',3',1''-terphenyl-5'-yl cyclopentylcarbamate (34). Yield: 149 mg, 73%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.60 (d, J = 7.6 Hz, 2H), 7.56 (s, 1H), 7.54 (d, J = 8.4 Hz, 2H), 7.47–7.38 (m, 5H), 7.34 (s, 1H), 7.30 (s, 1H), 5.06 (d, J = 7.2 Hz, 1H), 4.12–4.04 (m, 2H), 2.08–1.98 (m, 2H), 1.78–1.61 (m, 4H), 1.57–1.47 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 152.2, 144.5, 141.2, 139.7, 137.7, 133.0, 129.2, 129.1, 128.8, 128.1, 127.6, 123.1, 119.9, 119.5, 53.4, 33.5, 23.9 ppm. Anal. ($C_{22}H_{22}ClNO_2$) C, H, N.

4,4''-Dichloro-1,1',3',1''-terphenyl-5'-yl cyclopentylcarbamate (35). Yield: 118 mg, 88%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.54 (s, 1H), 7.52 (d, J = 8.0 Hz, 4H), 7.40 (d, J = 8.0 Hz, 4H), 7.30 (s, 2H), 5.06 (d, J = 7.2 Hz, 1H), 4.12–4.04 (m, 2H), 2.10–1.99 (m, 2H), 1.78–1.60 (m, 4H), 1.57–1.47 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 152.2, 142.2, 139.0, 134.2, 129.3, 128.8, 122.8, 119.8, 53.4, 33.5, 23.9 ppm. Anal. ($C_{24}H_{21}Cl_2NO_2 \cdot 0.5 H_2O$) C, H, N.

3,4-dichloro-1,1',3',1''-terphenyl-5'-yl cyclopentylcarbamate (36). Yield: 130 mg, 87%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.45 (d, J = 7.6 Hz, 1H), 7.65–7.52 (m, 3H), 7.50–7.34 (m, 6H), 7.28 (s, 1H), 5.07 (d, J = 6.4 Hz, 1H), 4.10–4.01 (m, 2H), 2.10–1.98 (m, 2H), 1.75–1.62 (m, 4H), 1.56–1.46 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 152.2, 140.5, 139.2, 137.7, 136.7, 132.5, 132.0, 131.1, 130.6, 129.7, 129.3, 128.5, 127.5, 123.1, 119.5, 119.4, 53.4, 33.5, 23.9 ppm. Anal. ($C_{24}H_{21}Cl_2NO_2 \cdot 0.5 H_2O$) C, H, N.

3,3'',4,4''-tetrachloro-1,1',3',1''-terphenyl-5'-yl cyclopentylcarbamate (37). Yield: 200 mg, 70%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.68 (d, J = 2.0 Hz, 2H), 7.54–7.49 (m, 2H), 7.46 (s, 1H), 7.43–7.38 (m, 2H), 7.34 (d, J = 1.0 Hz, 2H), 5.07 (d, J = 7.2 Hz, 1H), 4.12–4.03 (m, 2H), 2.09–2.01 (m, 2H), 1.78–1.62 (m, 4H), 1.57–1.47 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 152.4, 141.1, 140.3, 133.3, 132.4, 131.1, 129.3, 126.8, 122.7, 120.3, 53.5, 33.5, 23.9 ppm. Anal. ($C_{24}H_{19}Cl_4NO_2 \cdot 0.5 H_2O$) C, H, N.

4-Methoxy-1,1',3',1''-terphenyl-5'-yl cyclopentylcarbamate (38). Yield: 220 mg, 68%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.66 (d, J = 7.2 Hz, 2H), 7.62 (s, 1H), 7.60 (d, J = 8.8 Hz, 2H), 7.48 (t, J = 7.2 Hz, 2H), 7.39 (t, J = 7.2 Hz, 1H), 7.33 (s, 2H), 7.01 (d, J = 8.8 Hz, 2H), 5.09 (d, J = 7.6 Hz, 1H), 4.17–4.10 (m, 2H), 3.92 (s, 3H), 2.09–1.99 (m, 2H), 1.76–1.62 (m, 4H), 1.56–1.47 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 156.5, 152.5, 141.5, 140.2, 138.7, 136.7, 129.0, 128.6, 127.9, 127.6, 122.9, 119.1, 119.0, 114.5, 55.7, 53.4, 33.5, 23.9 ppm. Anal. ($C_{25}H_{25}NO_3$) C, H, N.

4,4''-Dimethoxy-1,1',3',1''-terphenyl-5'-yl cyclopentylcarbamate (39). Yield: 142 mg, 86%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.56 (s, 1H), 7.54 (d, J = 8.8 Hz, 4H), 7.24 (s, 2H), 6.90 (d, J = 8.8 Hz, 4H), 5.02 (d, J = 7.6 Hz, 1H), 4.12–4.04 (m, 2H), 3.85 (s, 6H), 2.09–2.01 (m, 2H), 1.77–1.62 (m, 4H), 1.57–1.48 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 158.2, 152.7, 140.2, 138.0, 132.2, 131.3, 128.7, 118.6, 114.5, 55.6, 53.5, 33.5, 23.9 ppm. Anal. ($C_{26}H_{27}NO_4$) C, H, N.

4-Methyl-1,1',3',1''-terphenyl-5'-yl cyclopentylcarbamate (40). Yield: 140 mg, 75%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.65–7.58 (m, 3H), 7.52 (d, J = 8.0 Hz, 2H), 7.43 (t, J = 7.6 Hz, 2H), 7.37–7.30 (m, 3H), 7.24 (d, J = 8.0 Hz, 2H), 5.06 (d, J = 7.6 Hz, 1H), 4.12–4.05 (m, 2H), 2.38 (s, 3H), 2.08–1.99 (m, 2H), 1.75–1.59 (m, 4H), 1.54–1.46 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 156.5, 152.0, 143.1, 140.8, 137.8, 137.7, 129.8, 129.0, 127.9, 127.6, 127.4, 123.0, 119.4, 119.3, 53.3, 33.5, 23.8, 21.4 ppm. Anal. ($C_{25}H_{25}NO_2$) C, H, N.

4,4''-Dimethyl-1,1',3',1''-terphenyl-5'-yl cyclopentylcarbamate (41). Yield: 215 mg, 82%. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.59 (s, 1H), 7.51 (d, J = 8.0 Hz, 4H), 7.29 (s, 2H), 7.24 (d, J = 8.0 Hz, 4H), 5.04 (d, J = 6.8 Hz, 1H), 4.12–4.03 (m, 2H), 2.39 (s, 6H), 2.08–1.98 (m, 2H), 1.77–1.60 (m, 4H), 1.57–1.47 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 152.0, 143.1, 137.9, 129.8, 127.4, 127.3, 122.9, 119.1, 53.3, 33.5, 23.9, 21.4 ppm. Anal. ($C_{26}H_{27}NO_2 \cdot 0.5\, H_2O$) C, H, N.

5.2.6 Synthesis of Cyclopentanecarboxylic acid [1,1',3',1'']terphenyl-5'-yl ester (26)

To a solution of cyclopentanecarboxylic acid (1.95 mmol, 0.18 mL) in dichloromethane, Et_3N (1.95 mmol, 0.27 mL) was added and stirred for 15 min. To it, EDAC (0.21 mmol, 400 mg) and HOBt (0.21 mmol, 290 mg) were added followed by [1,1',3',1'']terphenyl-5'-ol (**6**) (1.62 mmol, 400 mg) and Et_3N (1.62 mmol, 0.23 mL). The reaction mixture was then stirred at ambient temperature for 24 h. Upon completion (TLC), the reaction mixture was diluted with dichloromethane (100 mL), washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude compound was purified by flash column chromatography (EtOAc/petroleum ether, 1:5) to afford the desired product as colorless solid (130 mg, 24%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.65 (s, 1H), 7.62 (d, J = 7.6 Hz, 4H), 7.44 (t, J = 7.6 Hz, 4H), 7.36 (t, J = 7.6 Hz, 2H), 7.28 (s, 2H), 3.07–2.98 (m, 1H), 2.08–1.96 (m, 4H), 1.83–1.75 (m, 2H), 1.71–1.62 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 152.0, 143.4, 140.6, 129.1, 128.0, 127.6, 123.7, 119.5, 44.2, 30.4, 26.2 ppm.

5.2.7 Synthesis of 1,3-Dibromo-5-nitro-benzene (28)

To a solution of sodium nitrite (2.07 g, 30 mmol) in concentrated sulfuric acid (20 mL) was added slowly a suspension of 2,6-dibromo-4-nitroaniline (**27**) (6 g, 20 mmol) in glacial acetic acid (74 mL), maintaining an internal temperature below 20 ^{o}C. After stirring for 30 min, the resulting diazonium salt was then slowly added to a suspension of cuprous oxide (0.24 g, 3 mmol) in 95% ethanol (55 mL). After stirring overnight, the mixture was quenched with water (10 mL). The organic layer was separated, washed with saturated $NaHCO_3$, dried over anhydrous Na_2SO_4, filtered, and concentrated to afford a brown solid. The aqueous layer from the extracts was diluted, in portions, into 4 times the volume of water, causing precipitation of additional product which was collected by suction filtration. The combined lot of crude product was purified by flash column chromatography (dichloromethane/petroleum ether, 3:2) to afford analytically pure compound **28** as pale yellow solid (2.4 g, 42%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.40 (s, 2H), 8.00 (s, 1H) ^{13}C NMR (100 MHz, $CDCl_3$): δ = 151.2, 140.4, 125.9, 123.8 ppm.

5.2.8 Synthesis of 5'-Nitro-[1,1',3',1'']terphenyl (29)

To a stirred solution of 1,3-dibromo-5-nitro-benzene (**28**) (5.33 mmol, 1.5 g) in toluene (50 mL), phenylboronic acid (16 mmol, 1.95 g), $(Ph_3P)_4Pd$ (5 mol.%, 310 mg), and 2 M aq. solution of Na_2CO_3 (16 mmol, 1.7 g) was added and refluxed for 16 h. The progress of the reaction was monitored by TLC. Upon completion, the reaction was cooled to ambient temperature, diluted with EtOAc (400 mL), washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude compound was purified by flash column chromatography (EtOAc/petroleum ether, 1:19) to yield compound **29** as lemon colored fluffy solid (800 mg, 54%).

5.2.9 Synthesis of [1,1',3',1'']Terphenyl-5'-ylamine (30)

A homogeneous solution of 3,5-diphenyl-nitrobenzene (**29**) (500 mg, 1.81 mmol) and anhydrous stannous chloride (1.72 g, 9 mmol) in EtOH (5 mL) was heated at 70 ^{o}C for 30 min under N_2. Upon cooling, the solvent was removed under reduced pressure and the residue diluted with excess 20–30% aqueous NaOH. The aqueous layer was extracted with dichloromethane (3 x 50 mL). The combined organic extracts were washed with brine, dried

over anhydrous $NaSO_4$, filtered, and concentrated under reduced pressure. The crude compound was purified by flash column chromatography (EtOAc/petroleum ether, 1:5) to afford colorless solid of analytically pure compound **30** (250 mg, 56%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.61 (d, J = 7.6 Hz, 4H), 7.42 (t, J = 7.2 Hz, 4H), 7.33 (t, J = 7.2 Hz, 2H), 7.20 (s, 1H), 6.95 (s, 2H), 3.78 (brs, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 147.4, 143.2, 141.7, 129.0, 127.7, 127.5, 117.3, 113.2 ppm.

5.2.10 Synthesis of Cyclopentanecarboxylic acid [1,1',3',1'']terphenyl-5'-yl amide (31)

To a solution of cyclopentanecarboxylic acid (1.47 mmol, 0.16 mL) in dichloromethane, Et_3N (1.47 mmol, 0.21 mL) and ethyl choroformate (1.47 mmol, 0.14 mL) was added and stirred for 30 min at 0 ^oC. To it, [1,1',3',1'']terphenyl-5'-yl amine (**30**) (1.22 mmol, 300 mg) and Et_3N (1.22 mmol, 0.17 mL) was added and stirred at ambient temperature for 6 h. Upon completion (TLC), the reaction mixture was diluted with dichloromethane (100 mL), washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude compound was purified by flash column chromatography (EtOAc/petroleum ether, 1:4) to afford the desired product as colorless solid (205 mg, 60%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.81 (s, 2H), 7.61 (d, J = 7.2 Hz, 4H), 7.53 (s, 1H), 7.41 (t, J = 7.4 Hz, 4H), 7.34 (t, J = 7.2 Hz, 2H), 2.75–2.69 (m, 1H), 2.01–1.86 (m, 4H), 1.84–1.72 (m, 2H), 1.64–1.55 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 142.8, 141.0, 139.3, 129.1, 127.9, 127.6, 122.1, 117.8, 47.2, 30.9, 26.4 ppm.

5.2.11 Synthesis of Cyclopentyl-3-[1,1',3',1'']terphenyl-5'-yl-urea (32)

Triethylamine (0.98 mmol, 0.13 μL) was added to a stirred solution of [1,1',3',1'']terphenyl-5'-yl amine (**30**) (0.81 mmol, 200 mg) in anhydrous dichloromethane and stirred at ambient temperature for 15 min. To it, cyclopentyl isocyanate (1.22 mmol, 138 μL) was added and stirred for another 18 h at ambient temperature. Upon completion (TLC), the reaction mixture was diluted with dichloromethane (100 mL), washed with water, brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The crude compound was purified by flash column chromatography (EtOAc/petroleum ether, 1:5) to obtain compound **32** (200 mg, 69%). The column purified carbamate was crystallized using dichloromethane and petroleum ether. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.72–7.58 (m, 6H), 7.49–7.31 (m, 7H),

5.82 (d, J = 6.4 Hz, 1H), 4.18–4.10 (m, 1H), 2.07–1.91 (m, 2H), 1.74–1.57 (m, 4H), 1.51–1.39 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 156.5, 142.5, 141.1, 138.2, 128.8, 127.5, 127.2, 120.2, 116.8, 49.0, 33.4, 23.6 ppm.

5.2.12 Synthesis of Cyclopentyl-carbamic acid biphenyl-3-yl ester (33)

This compound was synthesized from commercially available 3-phenylphenol using the general method for carbamate synthesis. Yield: 279 mg, 81%. ^{1}H NMR (400 MHz, $CDCl_3$): δ =7.57 (d, J = 7.2 Hz, 2H), 7.47–7.39 (m, 4H), 7.37–7.31 (m, 2H), 7.11 (s, 1H), 4.10–4.02 (m, 1H), 2.08–1.98 (m, 2H), 1.70–1.56 (m, 4H), 1.52–1.46 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 142.8, 138.2, 129.7, 129.0, 128.9, 127.8, 127.7, 127.4, 124.1, 120.6, 53.2, 33.4, 23.8 ppm.

5.2.13 Biology - Materials

Org 43553, recLH and rec-hCG were provided by Schering Plough (Oss, The Netherlands); Org 43553 was synthesized as described previously.[211] Bovine serum albumin (BSA, fraction V) was purchased from Sigma (St. Louis, MO, U.S.A.), whereas BCA protein assay reagent was from Pierce Chemical Company (Rockford, IL, U.S.A.). [^{3}H]Org 43553 (16.6 Ci/mmol) was labeled as described in *Chapter 4*. ^{125}I-hCG (4408 Ci/mmol) was purchased from Perkin Elmer Life Sciences Inc. (Boston, MA, U.S.A.). Chinese Hamster Ovary (CHO-K1) cells stably expressing the human luteinizing hormone (LH) receptor and cAMP-response-element luciferase reporter gene (CRE-luc) were kindly provided by Schering-Plough (Oss, The Netherlands). All other chemicals and cell culture materials were obtained from standard commercial sources.

5.2.14 Cell Culture and Membrane Preparation

CHO cells with stable expression of the human LH receptor and CRE-luc (CHOhLHr_luc) were grown in culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F12 medium (1:1) supplemented with 7.5% normal adult bovine serum, streptomycin (100 µg/mL), penicillin (100 IU/ mL) at 37 °C in 5% CO_2. The

cells were subcultured twice weekly at a ratio of 1:20. Cell membranes were prepared as described in *Chapter 4*.

5.2.15 Radioligand Displacement Assays

^{125}I-hCG displacement assays were performed as described in *Chapter 4*. For [^{3}H]Org 43553, membrane aliquots containing 50 µg protein were incubated in a total volume of 100 µL assay buffer (25 mM Tris-HCl, pH 7.4, supplemented with 2 mM $MgCl_2$ and 0.1% BSA) at 30 °C for 90 min. Displacement experiments were performed using 10 µM or a range of concentrations of competing ligand in the presence of 4.5 nM [^{3}H]Org 43553. Non-specific binding was determined in the presence of 10 µM Org 43553 and represented approximately 35% of the total binding. [^{3}H]Org 43553 did not bind specifically to membranes prepared from CHO cells lacking the LH receptor. 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 1 mL ice-cold Tris-HCl buffer. Bound from free radioligand was immediately separated by rapid filtration through Whatman GF/B filters using a Millipore manifold. Filters were subsequently washed three times with ice-cold wash buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM $MgCl_2$ and 0.05% BSA). Filter-bound radioactivity was determined by scintillation spectrometry (Tri-Carb 2900TR; PerkinElmer Life and Analytical Sciences) after addition of 3.5 mL of PerkinElmer Emulsifier Safe.

5.2.16 Radioligand Dissociation Assays

Dissociation assays with ^{125}I-hCG were performed as described in *Chapter 4*. The amount of radioligand still bound to the receptor was measured after 4 h of dissociation. The obtained amount of radioligand binding determined at control conditions (t = 0) was set at 100%. For [^{3}H]Org 43553, dissociation experiments were performed by preincubating membrane aliquots containing 50 µg protein in a total volume of 100 µL assay buffer (25 mM Tris HCl, pH 7.4, supplemented with 2 mM $MgCl_2$ and 0.1% BSA) with 4.5 nM [^{3}H]Org 43553 at 30 °C for 90 min. After preincubation, dissociation was initiated by addition of 10 µM Org 43553 in the absence (control) or presence of allosteric modulators in a total volume of 5 µL of which was 50% (v/v) DMSO. The amount of radioligand still bound to the receptor was

measured after 30 min of dissociation. The obtained amount of radioligand binding determined at control conditions was set at 100%. In addition, the amount of [^{3}H]Org 43553 still bound to the receptor was measured at various time intervals for a total of 120 min in the absence (control) and presence of 1 and 10 µM **24**. Incubations were terminated and samples were obtained and analyzed as described under *Radioligand Displacement Assays.*

5.2.17 Luciferase Assays

CHOhLHr_luc cells were grown as described above. On the day of the assay, cells were washed with PBS and then harvested using trypsol (0.25% (w/v) in PBS containing 4.4 mM EDTA). Cells were resuspended in assay medium consisting of DMEM and F12 (1:1) supplemented with 1 µg/mL insulin and 5 µg/mL apo-transferrin. Typically, a well contained 30 µL of ligand, 30 µL of assay medium with or without 10 µM **24** and 30 µL cell suspension containing 7.5×10^5 cells/mL. Luciferase assays were performed using ten concentrations of recLH or Org 43553. Basal activity was determined in the presence of assay medium and represented approximately 10% of the maximal activity. Maximal receptor activity was determined in the presence of 1 nM recLH and was set at 100% in all experiments, whereas basal activity was set at 0% in all experiments. After 4 h of stimulation, 45 µL of Britelite® (PerkinElmer, Groningen, The Netherlands) was added to each well for detection of luciferase protein. Finally, the luminescence signal was quantified on a Microbeta Trilux 1450 Luminescence Counter (PerkinElmer, Groningen, The Netherlands).

5.2.18 LogD Determination by HPLC

Distribution coefficients (log D) were determined as described by Lombardo and coworkers.[278] In short, retention times of the compounds were determined in an HPLC system with three different methanol percentages. These retention times were converted to k' values by using the formula $k' = (t_r - t_0)/t_0$ in which t_r is the retention time and t_0 the retention time of an 'non-delayed' compound (pure methanol). The calculated k' values were plotted against the methanol percentage and extrapolated to a 0% methanol situation which yielded the k'w value (y axis cutoff). In a standard curve, the known logD values of the reference compounds were plotted against their k'w values found in the HPLC system used. From this standard curve the logD values of the compounds described in this paper were determined.

5.2.19 Data Analysis

All binding data were analyzed using the non-linear regression curve-fitting program GraphPad Prism v. 5.00 (GraphPad Software Inc, San Diego, CA, U.S.A.). Dissociation constants, k_{off}, were obtained by computer analysis of the exponential decay of [^{3}H]Org 43553 bound to the receptor. All values obtained are means of at least three independent experiments performed in duplicate.

5.3 RESULTS AND DISCUSSION

5.3.1 Chemistry

A series of symmetrical and unsymmetrical terphenyl carbamates was synthesized as depicted in Scheme 5.1. The synthesis was started from commercially available 3,5-dibromophenol **5**. The microwave assisted Suzuki-Miyaura cross coupling of phenol **5** with phenylboronic acid and substituted phenylboronic acid using the catalyst $[(Ph_3P)_4]Pd$ gave terphenylphenols **6–10**.[279] These compounds (**6–10**) were then treated with various isocyanates and Et_3N in anhydrous dichloromethane to furnish the terphenyl carbamates (**4**, **16–25**, **35**, **37**, **39**, **41**). The esterification of terphenylphenol **6** was achieved using EDAC and HOBt to afford the cyclopentyl ester **26**. The unsymmetrical terphenylphenols (**12–15**) were synthesized via the sequential Suzuki-Miyaura cross coupling of phenol **6** with phenylboronic acid followed by substituted phenylboronic acid. The intermediates were treated with cyclopentyl isocyanate and Et_3N in anhydrous dichloromethane to afford the terphenyl carbamates (**34**, **36**, **38**, **40**).

Scheme 5.1 Synthetic route to compounds **4**, **6**, **16**-**26**, **34**-**41**. a) $PhB(OH)_2$, aq. Na_2CO_3, toluene, MW, 10 min., 70-90%; b) EDAC, HOBt, cyclopentanecarboxylic acid, Et_3N, CH_2Cl_2, RT, 24 h, 40%; c) R^5NCO, Et_3N, CH_2Cl_2, RT, overnight, 70-90%; d) $PhB(OH)_2$, aq. Na_2CO_3, toluene, reflux, 3h, 30%; e) substituted phenylboronic acid, aq. Na_2CO_3, toluene, MW, 10 min., 35-52%; f) R^5NCO, Et_3N, CH_2Cl_2, RT, overnight, 60-82%.

Terphenyl amide derivative **31** and terphenyl urea derivative **32** were synthesized as outlined in Scheme 5.2. Deamination of commercially available 2,6-dibromo-4-nitroaniline **27** was achieved using $NaNO_2$ and CuO to yield 3,5-dibromonitrobenzene **28**.[280] The Suzuki-Miyaura cross coupling of **28** with phenylboronic acid using the catalyst $[(Ph_3P)_4]Pd$ gave 3,5-diphenylnitrobenzene **29**. The nitro group was reduced using anhydrous $SnCl_2$ in EtOH at 70 °C to afford amine **30** which subsequently was treated with cyclopentanecarboxylic acid, ethyl chloroformate and Et_3N in anhydrous dichloromethane to afford the amide **31**. Terphenyl urea **32** was synthesized from amine **30** using cyclopentyl isocyanate and Et_3N in anhydrous dichloromethane. Biphenyl carbamate **33** was synthesized by treating commercially available 3-phenylphenol with isopentyl isocyanate and Et_3N in anhydrous dichloromethane.

Scheme 5.2 Synthetic route to compounds **30**-**33**. Reagents and conditions: a) $NaNO_2$, H_2SO_4, CH_3COOH, CuO, EtOH, overnight, 30%; b) $(Ph_3P)_4Pd$, $PhB(OH)_2$, aq. Na_2CO_3, toluene, reflux, 16 h, 42%; c) anhydrous $SnCl_2$, EtOH, 70 °C, 30 min., 40%; d) cyclopentanecarboxylic acid, EtOCOCl, Et_3N, CH_2Cl_2, RT, 4 h, 50%; e) cyclopentyl isocyanate, Et_3N, CH_2Cl_2, RT, overnight, 70%.

5.3.2 Structure-Activity Relationships

In an initial screen, 50 diverse low molecular weight compounds were tested for their ability to either increase or decrease the dissociation rate of [^{3}H]Org 43553 from the human LH receptor stably expressed on CHO cell membranes. Most compounds did not change the dissociation rate compared to control conditions. However, some hits were obtained that significantly increased the dissociation rate of the radioligand, indicative for allosteric inhibition. In Figure 5.1, four of these hits (**1**-**4**) are depicted that showed some resemblance in their chemical structures. The presence of these compounds resulted in an increase of the dissociation by 42, 16, 29 and 79% when compared to the dissociation of [^{3}H]Org 43553 by unlabeled Org 43553 alone, respectively.

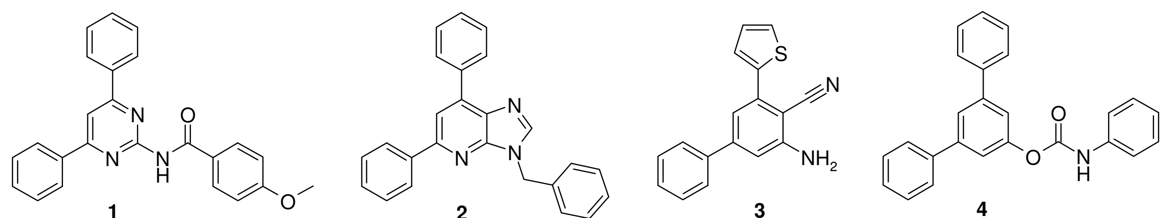

Figure 5.1 Chemical structures of the first hits (**1** – **4**) resulting from screening in the [^{3}H]Org 43553 dissociation assay. The presence of 10 μM of compounds **1** - **4** resulted in 42, 16, 29 and 79% enhanced radioligand dissociation compared to control conditions, respectively.

Subsequently, the structure-activity relationships around compound **4** were further explored for two reasons; 1) this was the most potent allosteric inhibitor (79% increased dissociation compared to control), and 2) compounds **1**-**3** have been reported as adenosine receptor antagonists, where **1** and **2** showed low affinity for the A_1 receptor subtype,[281,282] while **3** displayed nanomolar affinity for both the A_1 and A_{2A} receptor.[283] Notably, **4** did not show any affinity for the adenosine receptor subtypes (data not shown). Compound **4** also caused displacement of [^{3}H]Org 43553 in equilibrium radioligand binding studies (see also Table 5.1). This probably results from non-competitive (allosteric) inhibition, as shown for other allosteric inhibitors, e.g. 5-(N,N-hexamethylene)amiloride (HMA) on the human GnRH receptor (*Chapter 3*).

Table 5.1 Displacement and allosteric modulation of [^{3}H]Org 43553 binding at the human luteinizing hormone receptors by 10 μM of compounds **4**, **6**, **16**-**26**, **30**-**32**

Compound	R	% Displacement [a]	% Allosteric Inhibition [b]
4		28 (23/33)	79 (77/81)
6	OH	-2 (-4/2)	27 (26/27)
16		9 (8/9)	19 (12/26)
17		58 (56/59)	35 (23/47)
18		-1 (-1/-1)	61 (58/64)
19		47 (39/54)	72 (67/76)
20		61 (60/61)	65 (60/69)
21		30 (29/31)	80 (77/83)
22		14 (12/15)	55 (49/60)
23		68 (62/73)	82 (78/87)
24 (**LUF5771**)		91 (89/93)	88 (84/92)

25		83 (83/83)	51 (48/54)
26		9 (6/12)	41 (38/44)
30	NH_2	9 (8/9)	42 (31/52)
31		30 (25/35)	56 (53/60)
32		49 (46/53)	70 (64/75)

[a] % Displacement of specific [^{3}H]Org 43553 binding from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes at 10 µM concentrations (n = 2, duplicate)

[b] % Enhanced dissociation [^{3}H]Org 43553 from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes in the absence (control; set at 0%) or presence of 10 µM of the compounds (n = 2, duplicate)

Analogues of **4** were synthesized for further exploration of the structure-affinity relationships of this prototype allosteric inhibitor of the luteinizing hormone receptor; their behavior in radioligand binding studies is reported in Tables 5.1 and 5.2. First the phenyl carbamic acid of **4** was removed yielding a phenol analog (**6**) that had low modulating potency and did not cause any displacement. Addition of a 4-chloro (**16**) or 4-methoxy (**17**) also resulted in a lower modulating potency. Apparently substitution of the phenyl is not tolerated in that binding pocket. In addition, the carbamic acid was substituted with several alkyls, where an ethyl (**18**) and isopropyl substituent (**22**) were less potent than **4**, but compounds bearing a propyl (**19**), butyl (**20**) or pentyl (**21**) substituent were equally potent in modulating [^{3}H]Org 43553 binding. Remarkably, compound **18** was not able to displace the radioligand. The first compounds that showed an increased potency (with respect to allosteric inhibition) possessed a tert-butyl (**23**) or cyclopentyl (**24**) substituent. The latter compound was able to increase the dissociation of [^{3}H]Org 43553 by 88%. In addition, 10 µM of **24** caused a similar amount of displacement compared to the percentage allosteric inhibition. The more bulky cyclohexyl group (**25**) resulted in a loss of modulatory potency, which indicated some steric hindrance in the binding pocket. Then the carbamic acid linker between the cyclopentyl and the terphenyl scaffold was examined. Replacement with an ester (**26**) or amide (**31**) resulted in a moderate potency. To examine whether this was due to the length of

the linker, a urea derivative was introduced (**32**). This compound had only a slightly decreased potency indicating that at least the length or the location of the nitrogen in the linker was important. The free amine (**30**) did not increase the dissociation rate, which was similar to the hydroxyl (**6**) analog.

It follows from Table 5.1 that a carbamate linker with a cyclopentyl substituent results in the most potent allosteric inhibitor. Therefore, other analogs were prepared where the terphenyl scaffold was substituted (Table 5.2). First, one of the phenyl rings was removed (**33**), still yielding an allosteric inhibitor, but much less lipophilic (Figure 5.2). Apparently, the third phenyl ring is not a prerequisite for high modulating potency. Subsequently, either one or both phenyl rings were substituted. As the molecule has a symmetry axis it does not matter where the single substituent is introduced. Introduction of one 4-chloro (**34**) results in a slightly reduced potency that is both reflected in the displacement and modulating potency compared to **24**. Substitution of the second phenyl ring with a 4-chloro atom (**35**) decreases its ability to displace the radioligand even further. The initial concentration of a compound in

Table 5.2 Displacement and allosteric modulation of [^{3}H]Org 43553 binding at the human luteinizing hormone receptors by 10 µM of compounds **33**-**41**

Compound	R[1]	R[2]	% Displacement [a]	% Allosteric Inhibition [b]
33	H	phenyl	81 (77/84)	75 (70/79)
34	phenyl	4-Cl-phenyl	77 (71/82)	71 (65/77)
35	4-Cl-phenyl	4-Cl-phenyl	55 (50/60)	ND
36	phenyl	3,4-diCl-phenyl	69 (64/71)	64 (57/72)
37	3,4-diCl-phenyl	3,4-diCl-phenyl	7 (6/8)	ND
38	phenyl	4-MeO-phenyl	67 (58/76)	83 (82/83)
39	4-MeO-phenyl	4-MeO-phenyl	17 (7/26)	31 (27/34)
40	phenyl	4-Me-phenyl	72 (67/74)	102 (97/106)
41	4-Me-phenyl	4-Me-phenyl	62 (58/66)	59 (57/61)

[a] % Displacement of specific [^{3}H]Org 43553 binding from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes at 10 µM concentrations (n = 2, duplicate)

[b] % Enhanced dissociation [^{3}H]Org 43553 from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes in the absence (control; 0%) or presence of 10 µM of the compounds (n = 2, duplicate)

ND: not determined

a dissociation assay is 10 fold higher than in a displacement assay. For certain compounds (**35**, and **37**) this resulted in solubility problems and their modulating potency could therefore not be determined. In general, the modulatory potency of this series of compounds seems to parallel their displacement properties. Extrapolation of this notion would indicate that **35** will be a poor allosteric inhibitor. A similar observation was done for the single (**36**) and double 3,4-dichloro (**37**) substituted compounds. Compound **36** had an intermediate modulating potency, while that was almost completely lost for **37** (based on the displacement values due to solubility problems). The first substituted compound that did not loose a significant amount of potency was compound **38** with a 4-methoxy group. A second 4-methoxy substituent (**39**), however, resulted in a substantial loss of potency. Introduction of a smaller 4-methyl substituent (**40**) resulted in a significant gain of modulating potency, to the extent that in the presence of **40** the radioligand had fully dissociated from the receptor after 30 min in comparison to control conditions. Double substitution with a 4-methyl group (**41**) resulted in a loss of potency. In short, although a single substituent only results in a small loss or even a gain of potency, double substitution resulted in a significant loss of potency when compared to compound **24**. Apparently, only one of the pockets that accommodate a phenyl ring has some space for a substituent, where the smallest substituent, a 4-methyl (**40**) results in the highest potency.

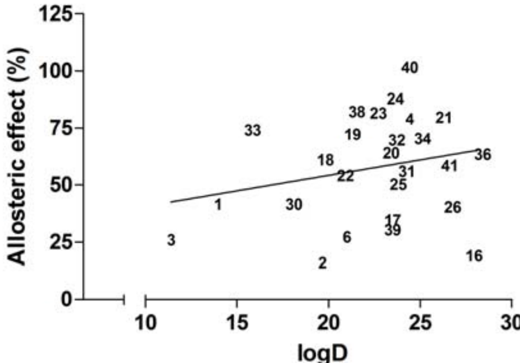

Figure 5.2 LogD of compounds **1-4**, **6**, **16-26**, **34-41** plotted against their allosteric effect on [^{3}H]Org 43553 binding. Analysis of the plot by linear regression resulted in a poor correlation (r^2 = 0.05791). Compound **35** and **37** did not dissolve in the methanol-based eluent and are therefore not included in the graph.

Due to the terphenyl scaffold, these ligands are highly lipophilic and hard to dissolve at high concentrations in an aqueous buffer. Therefore, the logD of these compounds was determined to assess whether a correlation between the lipophilicity (logD) of these compounds and their modulating behavior existed (Figure 5.2), which could indicate a non-specific effect. However, a poor correlation between the logD and the allosteric effect was found for the terphenyl compounds. The observed effect is therefore most probably truly LH receptor-mediated (see also the functional assays described below). This selectivity was further corroborated by the observation that LUF5771 (**24**) did not allosterically modulate another class A GPCR, the adenosine A_3 receptor (data not shown).

Table 5.3 Dissociation (k_{off}) rate constants of [^{3}H]Org 43553 in the presence of buffer (control), 1 μM or 10 μM LUF5771 (**24**).

Condition	k_{off} (min^{-1})[a]	Shift[b]
Control	0.026 ± 0.0007	-
+ 1 μM LUF5771	0.054 ± 0.004	2.1
+ 10 μM LUF5771	0.087 ± 0.002	3.3

[a] The values of the kinetic dissociation rate constants were obtained by analysis of the exponential dissociation of [^{3}H]Org 43553 bound to human luteinizing hormone receptors.
[b] The shift is defined as the ratio of k_{off} values in absence (control) and presence of LUF 5771 (**24**), respectively.
Values are means (± S.E.M.) of three separate assays performed in duplicate.

Further studies were undertaken to investigate the pharmacological characteristics of these novel allosteric inhibitors of the LH receptor. Based on the results of the first series of compounds (Table 5.1), the kinetic behavior of [^{3}H]Org 43553 was studied by performing full dissociation experiments in the absence (control) and presence of 1 μM or 10 μM **24** (Table 5.3 and Figure 5.3). The dissociation rate of the radioligand obtained by the addition of unlabeled ligand alone was 0.026 ± 0.0007 min^{-1}. This was slightly higher than the rate reported in *Chapter 4* (0.021 min^{-1}), due to the presence of a higher concentration of DMSO (also applied in the control experiment) that was necessary to dissolve these highly lipophilic compounds. In the presence of 1 μM **24** the dissociation rate was increased 2.1-fold to 0.054 ± 0.004 min^{-1}. In addition, allosteric inhibition by **24** was concentration-dependent, as the presence of 10 μM of **24** increased the dissociation even further, by 3.3-fold (Table 5.3). As

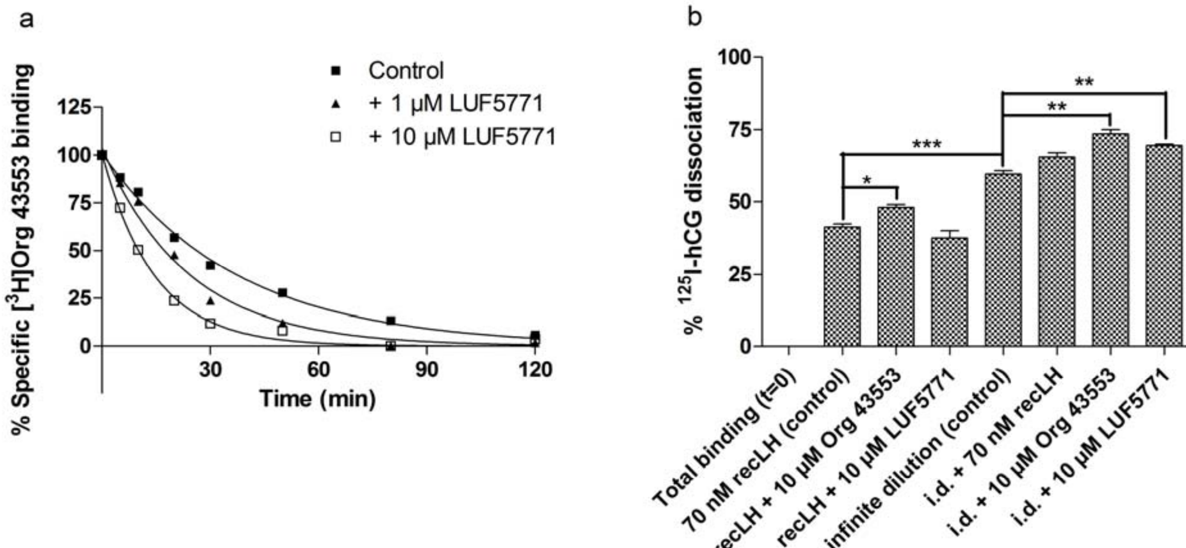

Figure 5.3 a) Dissociation kinetics of [^{3}H]Org 43553 binding to human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes. Dissociation was either initialized by the addition of 10 μM Org 43553 mixed with buffer (control), 1 μM or 10 μM (final concentrations) of LUF5771 (**24**). Representative graphs are shown from one experiment performed in duplicate (see Table 5.3 for kinetic parameters). b) Dissociation kinetics of ^{125}I-hCG binding to human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes. Dissociation was either initialized by the addition of 70 nM recLH mixed with buffer (control), 10 μM (final concentrations) Org 43553 or LUF5771. In addition, dissociation was initialized by infinite dilution (i.d.) in absence (control) or presence of 70 nM recLH, 10 μM Org 43553 or LUF5771. Values are means (± S.E.M.) of at least two separate assays performed in triplicate (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ versus control).

shown in the single point experiments **24** also displaced [^{3}H]Org 43553 in an equilibrium binding experiment by 91% at 10 μM (Table 5.1). Therefore, displacement of [^{3}H]Org 43553 equilibrium binding at different concentrations of **24** was determined (Figure 5.4). The obtained inhibition curve was best described by a one-site receptor model and yielded an IC_{50} value of 2.3 ± 0.4 μM with a pseudo-Hill coefficient of 1.1 ± 0.06.

The question arose if the LH receptor could possibly contain two allosteric sites in the 7-TM domain, as its orthosteric ligand binding site is located on the large N terminal domain of the receptor. For several class A GPCRs two binding sites, one orthosteric and one allosteric, have been reported in the 7-TM domain. The adenosine A_1 and A_3 receptors and the

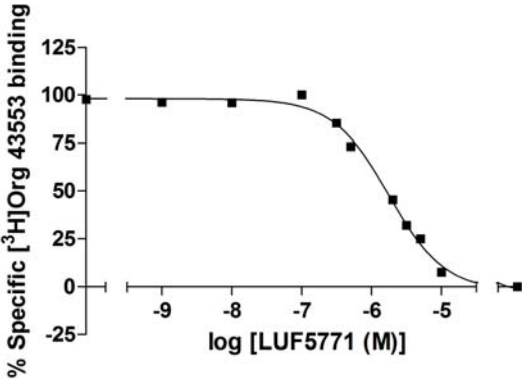

Figure 5.4 Displacement of [^{3}H]Org 43553 from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes by LUF 5771 (**24**). The IC_{50} value was 2.3 ± 0.4 µM. Representative graph is shown from one experiment performed in duplicate.

cannabinoid CB_1 receptor, where PD81,723, LUF6000 and Org27569 are selective allosteric enhancers, respectively, are typical examples.[239,284,285] Therefore, the effect of **24** on the equilibrium binding and dissociation kinetics of the iodinated endogenous ligand, ^{125}I-hCG, was examined. Compound **24** was not able to displace the hormone (data not shown), similar to Org 43553's behavior (*Chapter 4*). This indicates that LUF5771 probably also binds to the 7-TM domain like Org 43553 does.[36] In addition, the dissociation kinetics of ^{125}I-hCG under several conditions were examined (Figure 5.3b). An excess (70 nM) of recLH was able to induce 40% dissociation of ^{125}I-hCG after 4h. In the presence of 10 µM Org 43553 the dissociation was significantly increased, in line with a recent observation by Van Koppen and coworkers.[36] However, when dissociation was induced by recLH in the presence of 10 µM LUF5771 (**24**), no significant increase was observed. We also studied ^{125}I-hCG dissociation induced by infinite dilution (Figure 5.3b). In this case, the presence of recLH did not alter the amount of dissociation, while the presence of Org 43553 or LUF5771 significantly increased radioligand dissociation. Taken together, this suggests that the high molecular weight ligand, hCG (and most likely recLH) and the low molecular weight ligands, Org 43553 and LUF5771, bind at three distinct sites, where both LMW ligands induce a conformational change that (negatively) modulates hCG binding to the receptor.

Finally, the effect of LUF5771 (**24**) on the activation of the LH receptor by both of its endogenous hormones was examined in cAMP-induced luciferase assays (Table 5.4 and Figure 5.5). RecLH had an EC_{50} value of 56 ± 8 pM in this functional assay, which is

comparable to data reported in *Chapter 4*. The other endogenous ligand, rec-hCG, had a similar potency as recLH (EC_{50} = 97 ± 10 pM), while it had an approximately 25% lower efficacy in our hands. The presence of 10 µM LUF5771 did not affecr the efficacy, while an approximately 3-fold decrease in potency was observed for both recLH and rec-hCG. This indicates once more that LUF5771 induces a conformational change in the receptor that is

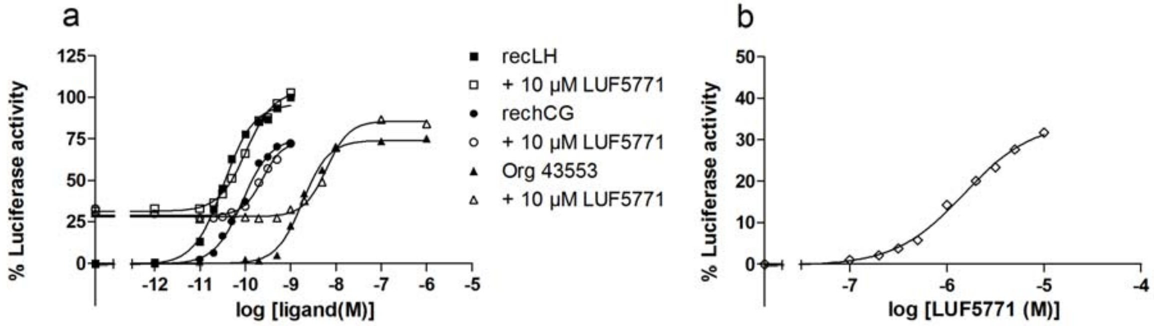

Figure 5.5 a) Concentration-effect curves of recLH (■), hCG (•) and Org 43553 (▲) in the absence (closed) or presence (open) of 10 µM LUF5771 and b) of LUF5771 itself for cAMP-mediated luciferase production through human luteinizing hormone receptors. Representative graphs from one experiment performed in duplicate (see Table 5.4 for EC_{50} and E_{max} values).

Table 5.4 Receptor activation by recLH, rec-hCG or Org 43553 in the presence or absence of 10 µM LUF5771 (**24**), expressed as EC_{50} and E_{max} values.

	Activity in luciferase assay[a]		
Compound	**EC_{50} (nM)**	**Shift[b]**	**E_{max} (%)[c]**
recLH	0.056 ± 0.008	-	100 ± 1
+ 10 µM LUF 5771	0.15 ± 0.02**	2.7	108 ± 4
rec-hCG	0.097 ± 0.01	-	74 ± 2
+ 10 µM LUF 5771	0.33 ± 0.05*	3.4	69 ± 3
Org 43553	1.9 ± 0.04	-	85 ± 4
+ 10 µM LUF 5771	4.6 ± 0.8*	2.4	92 ± 5

[a] cAMP-mediated luciferase activity in CHO-K1 cells that stably express the human luteinizing hormone receptor and CRE-luciferase reporter gene.
[b] The shift is defined as the ratio of EC_{50} values in the presence or absence of LUF5771, respectively.
[c] Maximal effect of either recLH or Org 43553 in the absence or presence of 10 µM LUF5771, where recLH in the absence of LUF5771 was set at 100%.
Values are means (± S.E.M.) of at least three separate assays performed in duplicate (* $p < 0.05$, ** $p < 0.005$ versus control).

disfavored by both endogenous hormones. As reported in *Chapter 4*, Org 43553 was a highly efficacious partial agonist in the cAMP-induced luciferase assay. Org 43553's efficacy was not affected by the presence of LUF5771, while its potency was decreased over 2-fold (EC_{50} = 4.6 ± 0.8 nM). The effect of two other compounds (**33** and **40**) from the second series (Table 5.2) was also investigated in a luciferase assay. Both **33** and **40** decreased the potency of recLH and Org 43553, similar to **24** (data not shown). From Figure 5.5a, it also follows that 10 µM of compound **24** alone was able to partially activate the LH receptor by 31 ± 4%. This agonistic behavior was further analyzed, and it was shown that **24** had an EC_{50} value of 1.6 ± 0.1 µM (Figure 5.5b). Some of the other terphenyl ligands also showed (low) intrinsic efficacy similar to compound LUF5771 (**24**; data not shown). It is noteworthy that the intrinsic activity of some of these terphenyl compounds might indicate that the second allosteric site is located close to or partially overlapping with the Org 43553 binding site. Interestingly, most allosteric modulators reported so far do not have an intrinsic efficacy at the receptor in the absence of an orthosteric agonist. However, there are some examples of (positive) allosteric modulators that can act as agonists by themselves.[49] For example, PD81,723 is an allosteric enhancer at the adenosine A_1 receptor as mentioned above, but it can also activate the receptor.[286] For the $GABA_B$ receptor CGP7930 was reported as an allosteric enhancer that is able to activate the receptor by interacting with the 7-TM domain of the $GABA_{B2}$ subunit.[287] Another example is AC-42 that was shown to active the receptor in absence of the orthosteric agonist, but it is also a modest allosteric enhancer of the muscarinic M_1 receptor.[288] For compounds that show allosteric agonism besides allosteric modulation of the orthosteric ligand, the term ago-allosteric modulator was proposed by Schwartz and coworkers.[289,290] To our knowledge only allosteric enhancers have been reported with intrinsic activity and no allosteric inhibitors. Compound **24** (LUF5771) could therefore be characterized as the first ago-allosteric inhibitor of the human LH receptor.

In conclusion, this paper describes the first series of allosteric inhibitors of [^{3}H]Org 43553 binding at the human LH receptor. In particular, LUF5771 (**24**) and **40** are highly potent. In addition, **24** inhibited the activation of the receptor by the endogenous ligand, recLH, and by Org 43553 in a functional assay. Although **24** is an allosteric inhibitor of recLH and Org 43553, it was also able to partially activate the LH receptor with low efficacy. The presence of a second allosteric site in the 7-TM domain, as demonstrated in this paper, may provide novel targets at the human luteinizing hormone receptor for low molecular weight allosteric modulators and allosteric agonists.