Substituted Terphenyl Compounds as the First Class of Low Molecular Weight Allosteric Inhibitors of the Luteinizing Hormone Receptor

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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.

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[a]).[1] 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.[2] 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.[3]

To increase patient convenience and compliance, efforts are made to develop nonpeptide orally active gonadotropins as drugs. For the LH receptor only a few compound classes have been described as LMW receptor agonists, such as the thienopyrimidine Org 43553. This compound was shown to have in vivo efficacy upon oral administration.[4] Recently the first high molecular weight antagonist was reported for the LH receptor, two fused beta-subunits of hCG.[5] However, LMW antagonists have not been reported so far.[6] Antagonists for the LH receptor may be novel contraceptive agents. In addition, antagonists could be used against ovarian cancer related to menopause.[7] 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.[8] 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.[9] 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**, which 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.

Results and Discussion

Chemistry. A series of symmetrical and unsymmetrical terphenyl carbamates was synthesized as depicted in Scheme 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**.[10] 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 **5** 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**).

Terphenyl amide derivative **31** and terphenyl urea derivative **32** were synthesized as outlined in Scheme 2. Deamination of commercially available 2,6-dibromo-4-nitroaniline **27** was

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[a] Abbreviations: BSA, bovine serum albumin; cAMP, cyclic adenosine-5′-monophosphate; CHO, Chinese hamster ovary; CHOhLHr_luc, human LH receptor and luciferase reporter gene transfected in CHO cells; CRE-luc, cAMP-response-element luciferase reporter gene; DCM, dichloromethane; DMF, dimethylformamide; EDAC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; EDTA, ethylene diamine tetraacetic acid; EtOAc, ethyl acetate; GPCR, G protein-coupled receptor; HOBt, 1-hydroxybenzotriazole; log *D*, logarithm of octanol−water distribution coefficient; Org 43553, 5-amino-2-methylsulfanyl-4-[3-(2-morpholin-4-yl-acetylamino)-phenyl]-thieno[2,3-*d*]pyrimidine-6-carboxylic acid *tert*-butylamide; $(Ph_3P)_4$Pd, tetrakis(triphenylphosphine)palladium; PBS, phosphate-buffered saline; rec-hCG, recombinant human chorionic gonadotropin; recLH, recombinant luteinizing hormone; SEM, standard error of the mean; TLC, thin-layer chromatography.

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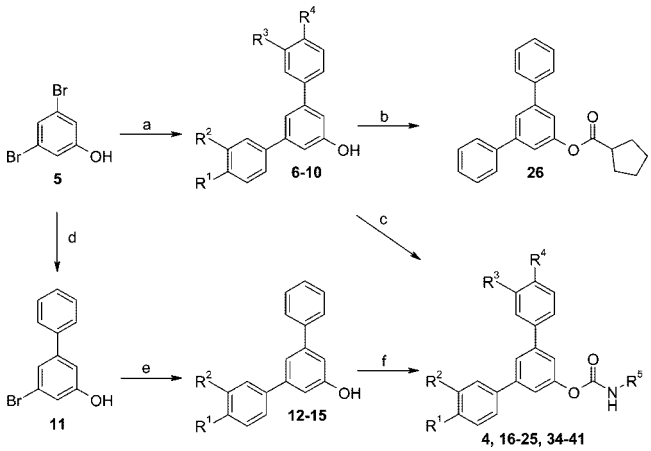
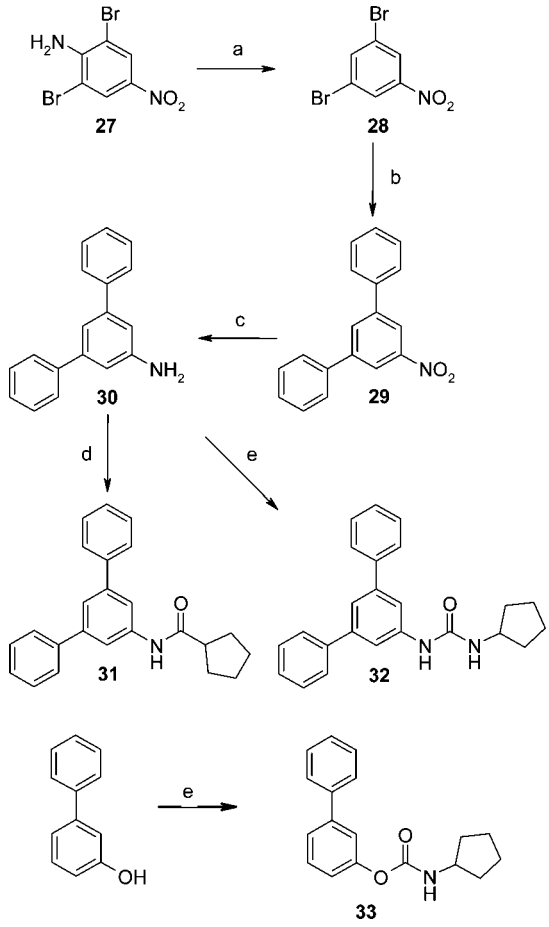
Scheme 1. Synthetic Route to Compounds **4**, **6**, **16**−**26**, **34**−**41**[a]



[a] Reagents and conditions: (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, 3 h, 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%.

Scheme 2. Synthetic Route to Compounds **30**−**33**[a]



[a] 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%.

achieved using $NaNO_2$ and CuO to yield 3,5-dibromonitrobenzene **28**.[11] 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.

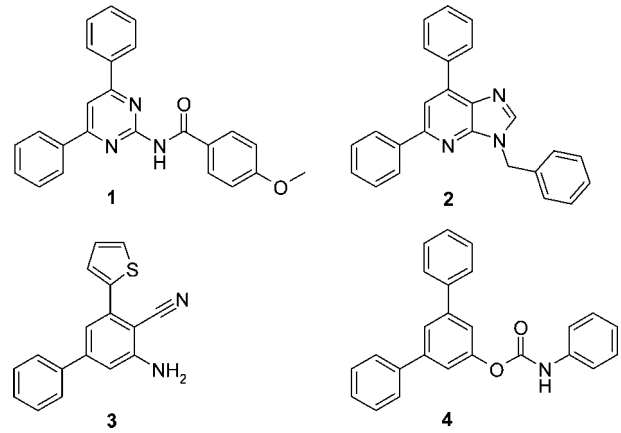


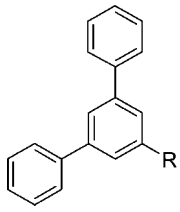
Figure 1. Chemical structures of the first hits (**1**−**4**) resulting from screening in the [^{3}H]Org 43553 dissociation assay. The presence of 10 μM of compounds **1**−**4** resulted in 42, 16, 29 and 79% enhanced radioligand dissociation compared to control conditions, respectively.

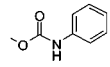
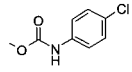
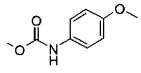
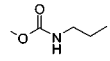
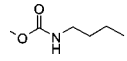
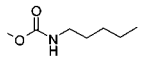
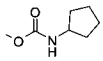
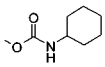
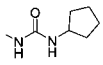
Structure−Activity Relationships. In an initial screen, 50 diverse low molecular weight compounds were tested for their ability to either increase or decrease the dissociation rate of [^{3}H]Org 43553 from the human LH receptor stably expressed on CHO cell membranes. Most compounds did not change the 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 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.

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,[12,13] while **3** displayed nanomolar affinity for both the A_1 and A_{2A} receptor.[14] Notably, **4** did not show any affinity for the adenosine receptor subtypes (data not shown). Compound **4** also caused a displacement of [^{3}H]Org 43553 in equilibrium radioligand binding studies (see also Table 1). This probably results from noncompetitive (allosteric) inhibition, as shown for other allosteric inhibitors, e.g. 5-(*N*,*N*-hexamethylene)amiloride (HMA) on the human GnRH receptor.[15]

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 1 and 2. First the phenyl carbamic acid of **4** was removed yielding a phenol analogue (**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

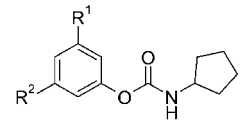
Table 1. Displacement and Allosteric Modulation of [^{3}H]Org 43553 Binding at the Human Luteinizing Hormone Receptor by 10 μM Concentration of Compounds **4**, **6**, **16**-**26**, **30**−**32**



compd	R	% displacement[a]	% allosteric inhibition[b]
4		28 (23/33)	79 (77/81)
6		-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		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 of [^{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 concentrations of the compounds (n = 2, duplicate).

Table 2. Displacement and Allosteric Modulation of [^{3}H]Org 43553 Binding at the Human Luteinizing Hormone Receptor by 10 μM Concentrations of Compounds **33**−**41**



compd	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[c]
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). [c] Not determined.

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 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 **24** caused a similar amount of displacement. The more bulky cyclohexyl group (**25**) resulted in a loss of 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**) analogue.

It follows from Table 1 that a carbamate linker with a cyclopentyl substituent results in the most potent allosteric inhibitor. Therefore, other analogues were prepared where the terphenyl scaffold was substituted (Table 2). First, one of the phenyl rings was removed (**33**), still yielding an allosteric inhibitor, but much less lipophilic (Figure 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 reflected in the displacement and modulating potency. 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 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. However, extrapolation of the other results indicates that the modulating potency of **35** will probably also be decreased. A similar observation was done for the single (**36**) and double 3,4-dichloro (**37**) substituted compounds. Compound **36** had an

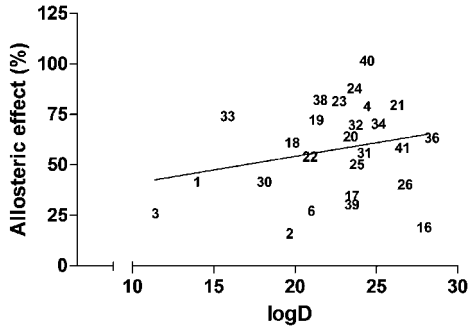


Figure 2. Log *D* of compounds **1**−**4**, **6**, **16**−**26**, **34**−**41** plotted against their allosteric effect on [^{3}H]Org 43553 binding. Analysis of the plot by linear regression resulted in a poor correlation ($r^2 = 0.05791$). Compounds **35** and **37** did not dissolve in the methanol-based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

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 lose 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.

Due to the terphenyl scaffold, these ligands are highly lipophilic and hard to dissolve in an aqueous buffer at high concentrations. Therefore, the log *D* of these compounds was determined to assess whether a correlation existed between the lipophilicity (log *D*) of these compounds and their modulating behavior (Figure 2), which could indicate a nonspecific effect. However, a poor correlation between the log *D* 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 compound **24** did not allosterically modulate another class A GPCR, the adenosine A_3 receptor (data not shown).

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 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 3 and Figure 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 previously (0.021 min^{-1}),[8] 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 3). As shown in the single point experiments **24** also displaced [^{3}H]Org 43553 in an equilibrium binding experiment by 91% at 10 μM (Table 1). Therefore, displacement of [^{3}H]Org 43553 equilibrium binding at different concentrations of **24** was determined (Figure 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.

Table 3. Dissociation (k_{off}) Rate Constants of [^{3}H]Org 43553 in the Presence of Buffer (Control) or 1 μM or 10 μM **24**[a]

condition	k_{off} (min^{-1})[b]	shift[c]
control	0.026 ± 0.0007	
+1 μM **24**	0.054 ± 0.004	2.1
+10 μM **24**	0.087 ± 0.002	3.3

[a] Values are means (±SEM) of three separate assays performed in duplicate. [b] 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. [c] The shift is defined as the ratio of k_{off} values in absence (control) and presence of compound **24**, respectively.

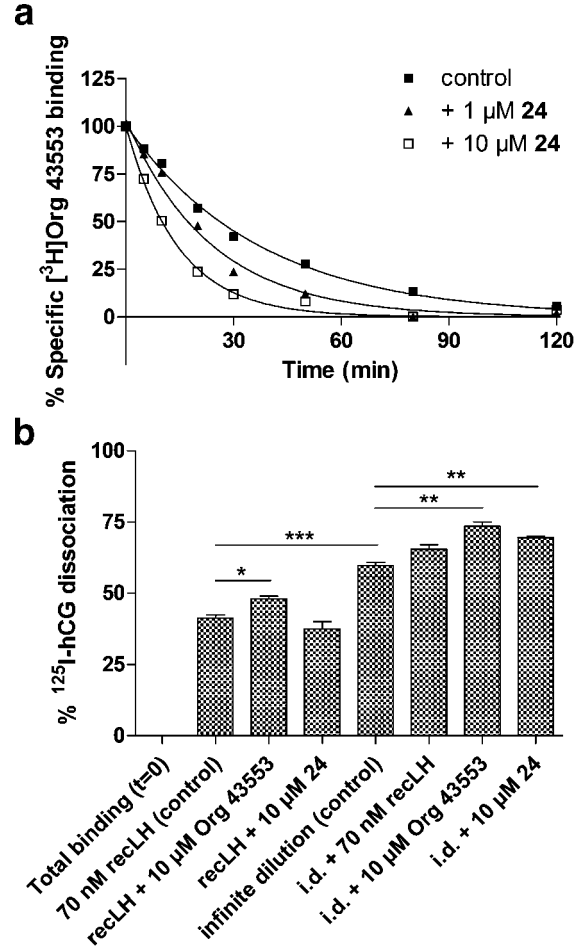


Figure 3. (a) Dissociation kinetics of [^{3}H]Org 43553 binding to human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes. Dissociation was initialized by the addition of 10 μM Org 43553 mixed with buffer (control) or 1 μM or 10 μM (final concentrations) of **24**. Representative graphs are shown from one experiment performed in duplicate (see Table 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 initialized by the addition of 70 nM recLH mixed with buffer (control) or 10 μM (final concentrations) Org 43553 or **24**. In addition, dissociation was initialized by infinite dilution (i.d.) in absence (control) or presence of 70 nM recLH, 10 μM Org 43553 or **24**. The amount of ^{125}I-hCG dissociation was determined after 4 h, where total binding (at $t = 0$) was set to 0% dissociation. Values are means (±SEM) of at least two separate assays performed in triplicate (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ versus control).

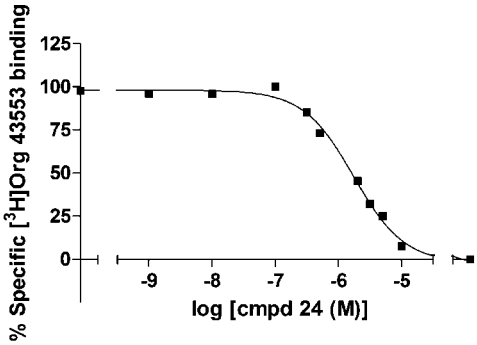


Figure 4. Displacement of [^{3}H]Org 43553 from human luteinizing hormone receptors stably expressed on CHO-K1 cell membranes by **24**. The IC_{50} value was 2.3 ± 0.4 μM. Representative graph is shown from one experiment performed in duplicate.

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 cannabinoid CB_1 receptor, where PD81,723, LUF6000 and Org27569 are selective allosteric enhancers, respectively, are typical examples.[16−18] Therefore, the effect of **24** on the equilibrium binding 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.[8] This indicates that **24** probably also binds to the 7-TM domain like Org 43553 does.[19] In addition, the dissociation kinetics of ^{125}I-hCG under several conditions were examined (Figure 3b). An excess (70 nM) of recLH was able to induce 40% dissociation of ^{125}I-hCG after 4 h. In the presence of 10 μM Org 43553 the dissociation was significantly increased, in line with a recent observation by Van Koppen and co-workers.[19] However, when dissociation was induced by recLH in the presence of 10 μM **24**, no significant increase was observed. We also studied ^{125}I-hCG dissociation induced by infinite dilution (Figure 3b). In this case, the presence of recLH did not alter the amount of dissociation, while the presence of Org 43553 or compound **24** 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 **24**, 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 compound **24** on the activation of the LH receptor by both of its endogenous hormones was examined in cAMP-induced luciferase assays (Table 4 and Figure 5). RecLH had an EC_{50} value of 56 ± 8 pM in this functional assay, which is comparable to previously published data.[8] 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 **24** did not affect the efficacy, while an approximately 3-fold decrease in potency was observed for both recLH and rec-hCG. This indicates that compound **24** induces a conformational change in the receptor that is disfavored by both endogenous hormones. As reported previously, Org 43553 was a highly efficacious partial agonist in the cAMP-induced luciferase assay (E_{max} = 85 ± 4%).[19] Org 43553's efficacy was not affected by the presence of compound **24**, while its potency was decreased over 2-fold (EC_{50} = 4.6 ± 0.8 nM). The effect of two other potent compounds (**33** and **40**) from the second series (Table 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).

Table 4. Receptor Activation by recLH, rec-hCG or Org 43553 in the Presence or Absence of 10 μM **24**, Expressed as EC_{50} and E_{max} Values[a]

	activity in luciferase assay[b]		
compound	EC_{50} (nM)	shift[c]	E_{max} (%)[d]
recLH	0.056 ± 0.008		100 ± 1
+10 μM 24	0.15 ± 0.02**	2.7	108 ± 4
Rec-hCG	0.097 ± 0.01		74 ± 2
+10 μM **24**	0.33 ± 0.05*	3.4	69 ± 3
Org 43553	1.9 ± 0.04		85 ± 4
+10 μM **24**	4.6 ± 0.8*	2.4	92 ± 5

[a] Values are means (±SEM) of at least three separate assays performed in duplicate (* $p < 0.05$, ** $p < 0.005$ versus control). [b] cAMP-mediated luciferase activity in CHO-K1 cells that stably express the human luteinizing hormone receptor and CRE-luciferase reporter gene. [c] The shift is defined as the ratio of EC_{50} values in the presence or absence of **24**, respectively. [d] Maximal effect of either recLH or Org 43553 in the absence or presence of 10 μM **24**, where recLH in the absence of **24** was set at 100%.

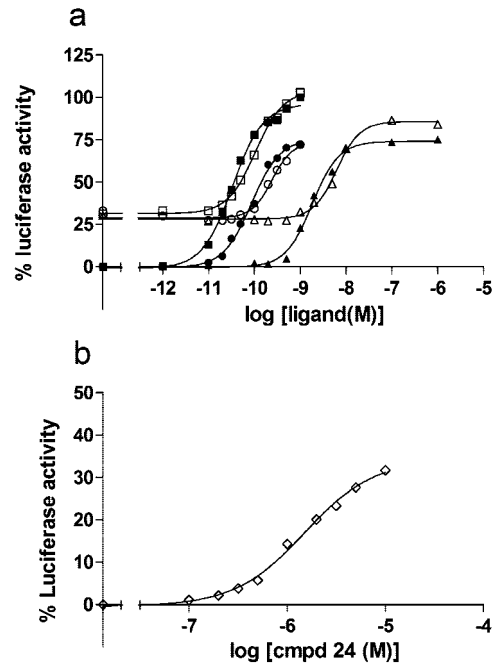


Figure 5. (a) Concentration−effect curves of recLH (squares), hCG (circles) and Org 43553 (triangles) in the absence (closed) or presence (open) of 10 μM **24** and (b) of **24** itself for cAMP-mediated luciferase production through human luteinizing hormone receptors. Representative graphs from one experiment performed in duplicate (see Table 4 for EC_{50} and E_{max} values).

Moreover, from Figure 5a it follows that 10 μM **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 5b). Some of the other terphenyl ligands also showed (low) intrinsic efficacy similar to compound **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.[20] For example, PD81,723 is an allosteric enhancer at the adenosine A_1 receptor as mentioned above, but it can also activate the receptor.[21] 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.[22] Another example is AC-42, which was shown to activate the receptor in absence of the orthosteric agonist, but it is also a

modest allosteric enhancer of the muscarinic M_1 receptor.[23] For compounds that show allosteric agonism besides allosteric modulation of the orthosteric ligand, the term ago-allosteric modulator was proposed by Schwartz and co-workers..[24,25] Compound **24** (LUF5771) could therefore be characterized as the first ago-allosteric inhibitor of the human LH receptor.

Conclusion

This paper describes the first series of allosteric inhibitors of [^{3}H]Org 43553 binding at the human LH receptor. In particular **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.

Experimental Section

Chemistry. Materials and Methods. 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.

General Procedure for Carbamate Synthesis. Triethylamine (1.5 equiv) was added to a stirred solution of phenol (1 equiv) in anhydrous dichloromethane and stirred at ambient temperature for 15 min. Isocyanate (1.5 equiv) 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, washed with 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%. Mp: 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.

Cyclopentyl-carbamic Acid [1,1′,3′,1″]Terphenyl-5′-yl Ester (24). Yield: 197 mg, 68%. Mp: 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.

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.

Biology. Materials and Methods. Org 43553, recLH and rec-hCG were provided by Schering Plough (Oss, The Netherlands); Org 43553 was synthesized as described previously.[26] Bovine serum albumin (BSA, fraction V) was purchased from Sigma (St. Louis, MO), whereas BCA protein assay reagent was from Pierce Chemical Company (Rockford, IL). [^{3}H]Org 43553 (16.6 Ci/mmol) was labeled as described previously.[8] ^{125}I-hCG (4408 Ci/mmol) was purchased from Perkin-Elmer Life Sciences Inc. (Boston, MA). 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.

Cell Culture and Membrane Preparation. CHO cells with stable expression of the human LH receptor and CRE-luc (CHOhL-Hr_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 previously.[8]

Radioligand Displacement Assays. ^{125}I-hCG displacement assays were performed as described previously.[8] For [^{3}H]Org 43553, membrane aliquots containing 50 μg of protein were incubated in a total volume of 100 μL of 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. Nonspecific 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 nonspecific binding was set at 0%. Incubations were terminated by dilution with 1 mL of 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.

Radioligand Dissociation Assays. Dissociation assays with ^{125}I-hCG were performed as described previously.[8] The amount of radioligand still bound to the receptor was measured after 4 h of dissociation. The total amount of radioligand binding determined at t = 0 was set to 0%, while nonspecific binding was set at 100%. For [^{3}H]Org 43553, dissociation experiments were performed by preincubating membrane aliquots containing 50 μg of protein in a total volume of 100 μL of 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.

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 of cell suspension containing 7.5×10^5 cells/mL. Luciferase assays were performed using ten concentrations of recLH, rec-hCG 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).

Log *D* Determination by HPLC. Distribution coefficients (log *D*) were determined as described by Lombardo and coworkers.[27] 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 a "nondelayed" 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 log *D* values of the reference compounds were plotted against their $k'w$ values found in the HPLC system used. From this standard curve the log *D* values of the compounds described in this paper were determined.

Data Analysis. All binding data were analyzed using the nonlinear regression curve-fitting program GraphPad Prism v. 5.00 (GraphPad Software Inc., San Diego, CA). 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.

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Supporting Information Available: Experimental details of the synthesis of the compounds described in this paper, their ^{1}H NMR and ^{13}C NMR spectroscopic data, and their elemental analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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