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# Identification, synthesis, and biological evaluation of novel pyrazoles as low molecular weight luteinizing hormone receptor agonists

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**Abstract**—In the course of a high throughput screening, a series of pyrazole compounds were identified with luteinizing hormone receptor (LH-R) agonist activity. A focused pyrazole library was produced by solid-phase synthesis and key pyrazole regioisomers were obtained selectively in solution. Evaluation of those compounds in a cAMP assay in CHO cells transfected with h-LH receptor allowed us to propose a structure–activity relationship model for this series and led to the identification of the first low molecular weight molecule with in vitro activity in a Leydig cells assay ($ED_{50}$ = 1.31 μM) and in vivo in a model of testosterone induction in rats (significant effect at 32 mpk ip).


Luteinizing hormone (LH), a member of the gonadotropin family, plays an essential role in human reproduction. In women, LH promotes the maturation of follicular cells in the ovary,[1] while in men, it is involved in spermatogenesis and stimulation of testosterone production in Leydig cells.[2] LH is a relatively large heterodimeric glycoprotein (28–38 kDa). Its receptor (LH-R) belongs to the super family of G protein-coupled receptors (GPCRs). The signal transduction pathway is essentially mediated by an increase in the level of cAMP through Gs protein. Like all other members of the gonadotropin hormone receptor family, LH receptor can be distinguished by an extremely long extracellular domain containing several leucine-rich repeats.[3] Although the binding mode of LH to its receptor is currently still not fully understood, it was demonstrated that LH accesses the receptor via its extracellular domain in the region of exons 1–8. With high affinity binding, LH could induce conformational changes in the extracellular loop of the receptor, activating the signal transduction cascade of the transmembrane region.[4,5]

LH therapy is currently utilized in conjunction with follicle stimulating hormone (FSH) to treat infertility and spermatogenesis disorders. Historically the gonadotropins FSH and LH were extracted from the urine of postmenopausal women. The LH activity of urinary hMG (human menopausal gonadotropin) was however primarily provided by addition of urinary human chorionic gonadotropin (hCG) due to the low amount of naturally occurring LH in postmenopausal urine. Recombinant or urinary gonadotropins are required to be injected parenterally, the availability of oral drugs would simplify the treatment regimen and increase the level of comfort for patients. In this paper, we report novel LMW (low molecular weight) LH-receptor agonists exhibiting in vivo activity in a model of testosterone induction in rat. To date, only few LMW modulators of gonadotropin receptors have been described[6–10] and only one example of a LH receptor agonist exhibiting in vivo activity has been reported so far.[11] A screening campaign was performed in our laboratory, resulting in the identification of a series of pyrazole positives. A focused library of pyrazoles was then synthesized by a

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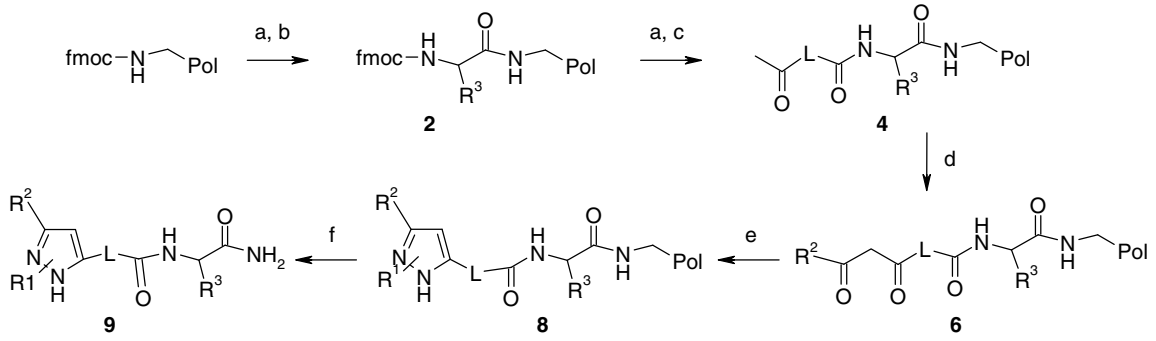
solid-phase synthesis (SPS) strategy. The preliminary structure–activity relationship (SAR) obtained from these pyrazoles was further refined by the synthesis of key compounds following the same solid-phase route. A new solution-phase route was developed in order to access the most active compounds in large amounts and with the desired regioselectivity. The best agonists were further evaluated in vitro in a cell assay measuring testosterone production and in vivo in a testosterone induction model in rats.

The pyrazole library was prepared on solid phase using a rink amide linker (Scheme 1). After deprotection of the linker, amino acids **1** were first loaded on the resin using standard coupling conditions and subsequently deprotected. The free amines were then coupled with keto-acids **3** possessing diverse spacers L such as alkyl or heteroalkyl chains or aryl or heteroaryl rings to give the aceto-derivatives **4**. β-Diketones **6** were obtained by reaction of intermediates **4** with aromatic or heteroaromatic esters **5** and treated with alkyl or aryl hydrazines **7** to form the pyrazole ring. After cleavage from the resin, a mixture of two regioisomers, 1,3,5- and 2,3,5-substituted pyrazoles, was isolated with overall yields higher than 90% in most examples. Both regioisomers could be isolated by preparative HPLC. Generally, the 2,3,5-substituted pyrazole is observed to be the major isomer with this synthetic method.[12] This was proven to be the case for this library as well, when the structure of compounds **10** and **11** was unambiguously assigned by nuclear Overhauser experiments on both regioisomers[13] (Fig. 1). As the SPS route gave too small quantities of the minor 1,3,5-isomer, a novel, regioselective route was developed. Very few regioselective pyrazole syntheses are described in the literature and most of them lead to the 2,3,5-substituted regioisomer. One new regiospecific approach for 1,3,5-substituted pyrazoles was recently proposed by Katzenellenbogen and co-workers,[14] using the cyclization of a hydrazone intermediate. However, when the hydrazone formed by condensation of 3-acetylpyridine and 4-*tert*-butylphenyl hydrazine was treated with propionic anhydride, the cyclization failed in all conditions, whether neutral, acidic or basic. Another regiospecific route to access 1,3,5-substituted pyrazoles was described by Murray.[15] He observed that the presence of a hydroxypropyl chain in the β-diketo precursor could reverse the usual regioselectivity of the pyrazole ring formation. Indeed, when applied to 3-pyridinyldiketone **12** and 4-*tert*-butyl hydrazine, those conditions led to the formation of 2,3,5-substituted pyrazole **13** and 1,3,5-substituted pyrazole **14** in a 2:8 ratio, respectively, with a good overall yield (Scheme 2). Pyrazole alcohol **14** was a key intermediate for the synthesis of numerous 1,3,5-substituted pyrazoles as illustrated by the synthesis of compound **10**. The mixture of the two alcohols **13** and **14** was oxidized to the corresponding aldehydes with Dess–Martin reagent. A two-carbon homologation through a Wittig–Horner reaction with *tert*-butyl diethylphosphonoacetate gave α,β-unsaturated esters **15** and **16**. Separation of the two regioisomers was achieved at this stage by flash chromatography. Reduction of the double bond, followed by acidic hydrolysis of the ester, led to the acid **17** in good yield. The final compound **10** was easily obtained from that intermediate by coupling with *O*-*tert*-butyl-L-tyrosinamide using standard coupling conditions and acidic hydrolysis of the *tert*-butyl ether.




**Scheme 1.** Solid-phase synthesis of pyrazoles. Reagents and conditions: (a) 20% piperidine in DMF, 25 °C, 2 h; (b) $HOOCCHR_3NHFmoc$ **1**, HATU, DIEA, DMF, 25 °C, 24 h; (c) HOOC-L-COMe **3**, HATU, DIEA, DMF, 25 °C, 24 h; (d) $R^2COOMe$ **5**, NaH, DMA, 85 °C, 24 h; (e) $R^1NHNH_2$ **7**, DIEA, DMA, 80 °C, 24 h; (f) TFA/DCM 2:1, 25 °C, 2 h.

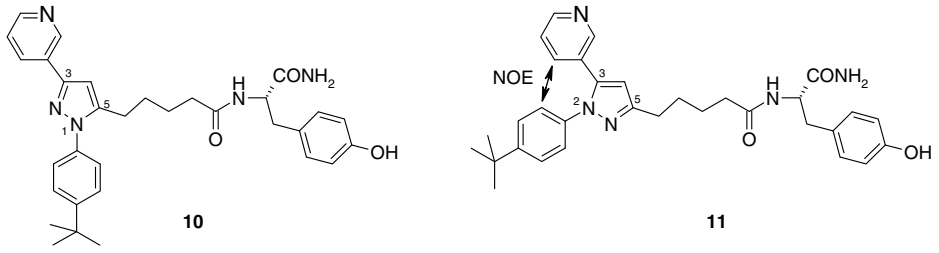
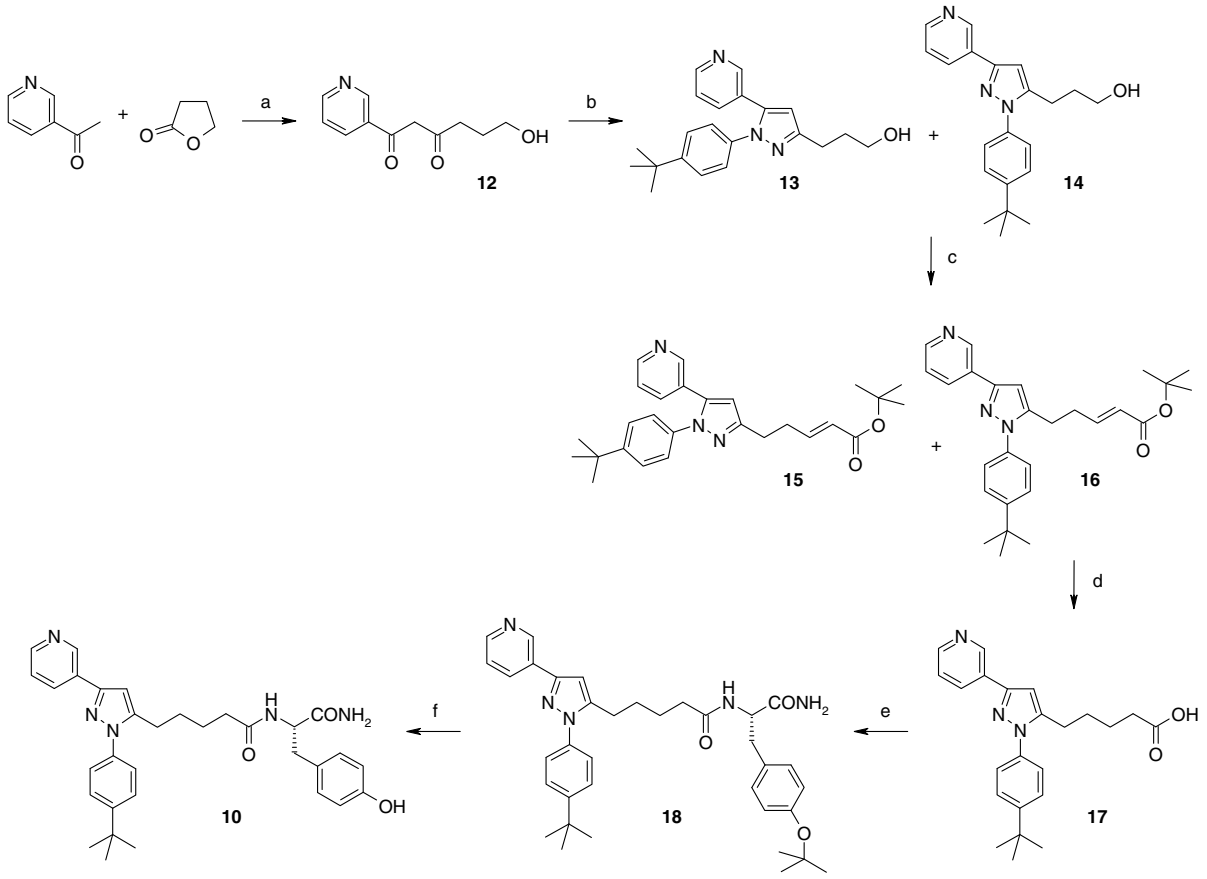



**Figure 1.** NOE cross peaks were observed between the *ortho*-protons of the pyridine ring and of the *tert*-butylphenyl ring for isomer **11** but not for isomer **10**.




**Scheme 2.** Solution-phase synthesis of pyrazoles. Reagents and conditions: (a) NaH, THF/DMSO, 25 °C, 24 h, 65%; (b) *p*-$^t$BuPheNHNH$_2$·HCl, 2 M HCl in ether, MeOH, 25 °C, 15 h, 87%; (c) i—Dess–Martin, DCM, 25 °C, 4 h, ii—(EtO)$_2$POCH$_2$COOtBu, NaH, THF, 0 °C, 1 h, 40%; (d) i—HCOONH$_4$, 10% Pd/C, MeOH, reflux, 1 h, ii—TFA/DCM, 25 °C, 4 h, 90%; (e) *O*-*tert*-butyltyrosinamide, EDC, HOBT, DIEA, 25 °C, 24 h, 80%; (f) TFA/DCM, 25 °C, 1 h, 82%.

The activity of compounds was assessed by an Amersham cAMP specific SPA assay using hLH-R CHO cells. The initial screening was designed to detect agonists: production of cAMP in response to a 12μM concentration of tested compound was measured. Compounds showing more than 60% of the maximal observed response normalized to the response obtained without stimulation were considered as positives. Purity of positives was controlled,[16] and $EC_{50}$ and efficacy were measured with dose–response curves for selected positives.[17] Specificity for hLHR was demonstrated by testing the compounds on untransfected CHO parental cells. The screening of the pyrazole library allowed us to define the main features required for the activity (Fig. 2): the nitrogen substituent of the pyrazole ring ($R^1$) has to be a large lipophilic group, such as 4-*tert*-butylphenyl or benzyl. Pentyl and heptyl chains were more active than phenyl substituted with small non-polar groups, like methyl, or polar groups, like Cl, COOH, OMe. For the substituent $R^2$ in position 3, pyridines and quinolines gave the best activities amongst a selection of aryl and heteroaryl groups. Lipophilic spacers L like alkyl chains or phenyl rings were well tolerated between the pyrazole ring and the amino acid moiety, but the presence of a heteroatom either in the chain or in a cycle led to a decrease in activity. More importantly, only

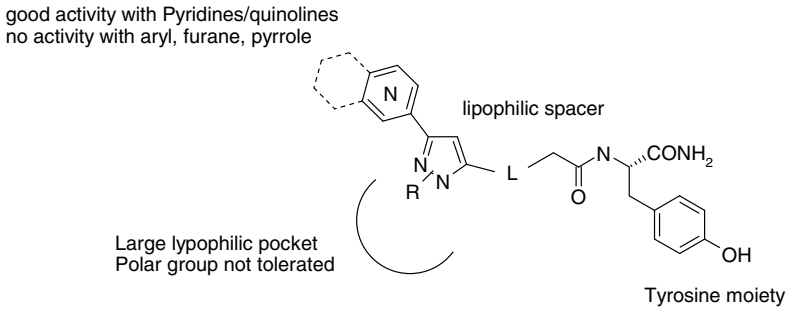



**Figure 2.** Preliminary SAR of the pyrazole series.

compounds with tyrosine as the amino acid moiety gave acceptable activity from a selection of nineteen diverse amino acids. As the screening was performed using a mixture of the two possible pyrazole regioisomers, refinement of the initial SAR could be achieved by the synthesis of some key compounds as single regioisomers. Compounds **19** and **20**, isolated as pure regioisomers (Fig. 3), were tested in the SPA assay. The minor regioisomer, the 1,3,5-substituted pyrazole **20** ($EC_{50}$ = 250 nM) was more active than the 2,3,5-substituted pyrazole **19** ($EC_{50}$ = 2.3 μM) and this observation entailed the necessity to optimize a route to access the minor 1,3,5-regioisomer in higher yield. Results obtained for representative 1,3,5-substituted pyrazoles are summarized in Table 1. A four-carbon alkyl chain spacer gives better activity compared to a shorter chain (compounds **21** and **10**), while a phenyl ring spacer seems to decrease the activity (isoquinoline **22**), as well as the presence of a heteroatom in the alkyl chain (compound **23**). The hydroxy substituent of the tyrosine moiety contributes to the high activity (compound **10** vs **24**). The distance between the pyrazole ring and the hydroxy residue appears to be more important for the activity than the arrangement of the spacer and the position of the amino acid, as illustrated by the activities found for compounds **25**, **26**, and **20**. The pyrazole substituent at position 3 can be a pyridine or a quinoline, although pyridines are more active than quinolines (data not shown) and 3-pyridine appears to be optimal in terms of potencies and efficacies compared to 2- and 4-pyridines (see compounds **20**, **10** and **26**, for example). Finally, the carboxamido group does not appear to be necessary and can be removed without altering the activity (compounds **27** and **28**).

Compound **10**, which displayed one of the best potency and efficacy, was selected for further pharmacological characterization. It was tested in a rat Leydig cells assay using testosterone as the end point measurement[18] and displayed an $ED_{50}$ of 1.31 ± 0.41 μM with a 78% efficacy. The lower activity in this bioassay (65-fold difference as compared to the cAMP assay in hLH-transfected CHO cells) may be explained by a species difference. However, such differences in potency are often observed between cellular assay using native cells and constructed cells.[9] In a binding assay, compound **10** was not able to displace $^{125}$I-labeled hCG while LH was measured with a $K_i$ = 311 pM in the same assay. Only one LMW

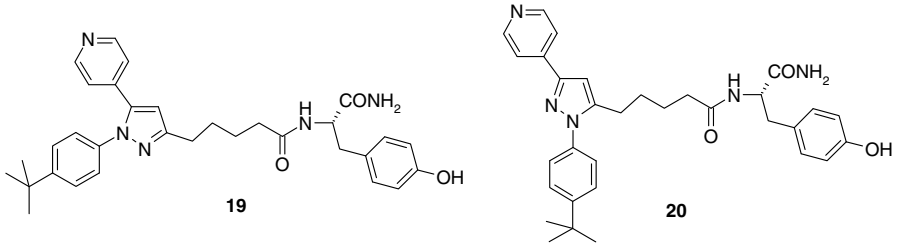
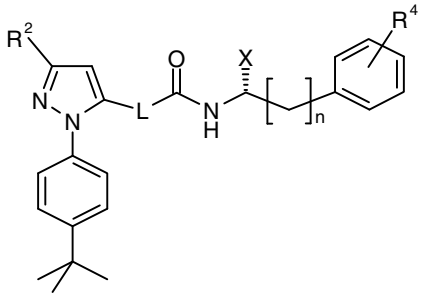



**Figure 3.**

**Table 1.** Refined SAR of the 1,3,5-pyrazoles series



| Compound | $R^2$ | L | X | $R^4$ | *n* | $EC_{50}$[a] (μM) (%) | Purity[b] |
|---|---|---|---|---|---|---|---|
| **10** | 3-Pyridine | $(CH_2)_4$ | $CONH_2$ | 4-OH | 1 | 0.02 ± 0.01 (70%) | 98 |
| **20** | 4-Pyridine | $(CH_2)_4$ | $CONH_2$ | 4-OH | 1 | 0.25 ± 0.04 (55%) | 99 |
| **21** | 3-Pyridine | $(CH_2)_2$ | $CONH_2$ | 4-OH | 1 | >100 | 92 |
| **22** | 3-Isoquinoline | 1,3-Ph | $CONH_2$ | 4-OH | 1 | 0.50 ± 0.10 (19%) | 77 |
| **23** | 4-Pyridine | $HN(CH_2)_3$ | $CONH_2$ | 4-OH | 1 | >100 | 94 |
| **24** | 3-Pyridine | $(CH_2)_4$ | $CONH_2$ | 4-O$^t$Bu | 1 | 12.1 ± 2.0 (72%) | 98 |
| **25** | 2-Pyridine | $(CH_2)_5$ | $CONH_2$ | 4-OH | 0 | 0.17 ± 0.02 (78%) | 96 |
| **26** | 2-Pyridine | $(CH_2)_5$ | $CONH_2$ | 3-OH | 1 | 0.15 ± 0.02 (41%) | 90 |
| **27** | 4-Pyridine | $(CH_2)_4$ | $CH_2CONH_2$ | 4-OH | 1 | 0.19 ± 0.06 (66%) | 88 |
| **28** | 3-Pyridine | $(CH_2)_4$ | H | 4-OH | 1 | 0.08 ± 0.01 (45%) | 98 |

[a] $EC_{50}$ (μM) in the CHO-hLHR-cAMP assay (% max LH response). Data are presented as means ± SE (*n* = 3).
[b] % purity determined by LC/MS.

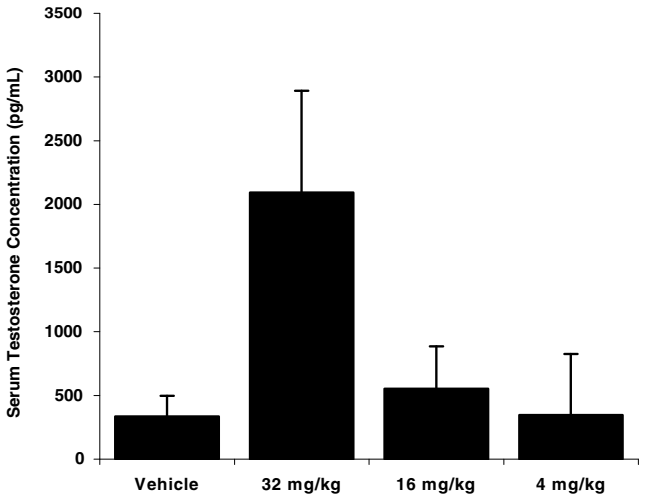



**Figure 4.** Two-month-old male rats testosterone-suppressed by a subcutaneous pellet of DES were injected with compound **10** (32, 16, and 4 mg/kg) or vehicle. Serum testosterone concentrations measured by radioimmunoassay.

modulator of gonadotropin receptors is described with the ability to displace the corresponding gonadotropin[6] while many others[10,11] do not. It is assumed that for most LMW modulators, the interaction with the receptor takes place directly in the transmembrane region of the receptor.

Finally, compound **10** was tested in a model of testosterone induction in rat.[19] As shown in Figure 4, when administered ip at doses between 4 and 32 mg/kg, compound **10** induced increased testosterone concentrations in a dose-dependent manner.

In summary, we have identified, synthesized, and optimized a new series of pyrazoles as LH receptor agonists. Compound **10**, one of the most potent and efficient agonist in the cAMP assay, revealed a promising biological profile in cellular and in vivo LH activation models. This compound is the first LH agonist, which showed in vivo activity in a testosterone induction model.

## Acknowledgments

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13. Compounds **10** and **11** were obtained in a 1:9 ratio and separated by reversed-phase HPLC using a DELTAPAK $C_{18}$ column with a linear gradient of 0.1% TFA water/acetonitrile 95:5 to 60:40 in one hour. Structures were assigned by Nuclear Overhauser experiments performed on a JEOL 400 MHz NMR apparatus. There were NOE cross peaks displayed between protons of the pyridine ring and protons of the *tert*-butylphenyl ring of isomer **11**, and no NOE effect observed between those protons for isomer **10**. Compound **11** (more polar): *N*-{5-[2-(4-*tert*-butylphenyl)-3-pyridin-3-yl-1*H*-pyrazol-5-yl]pentanoyl}-L-tyrosinamide trifluoroacetate salt, $^1$H NMR (400 MHz, DMSO): $\delta$ = 1.28 (s, 9H), 1.55 (m, 4H), 2.12 (t, $J$ = 7.3 Hz, 2H), 2.63 (m, 3H), 2.86 (dd, $J$ = 13.9, 5.3 Hz, 1H), 4.35 (m, 1H), 5.60 (br s, 1H), 6.61 (s, 1H), 6.62 (d, $J$ = 8.8 Hz, 2H), 6.98 (m, 1H), 7.01 (d, $J$ = 8.8 Hz, 2H), 7.19 (d, $J$ = 8.8 Hz, 2H), 7.36 (m, 1H), 7.42 (d, $J$ = 8.8 Hz, 2H), 7.51 (dd, $J$ = 8.1, 5.1 Hz, 1H), 7.75 (dt, $J$ = 8.1, 2.2 Hz, 1H), 8.52 (dd, $J$ = 2.2, 0.7 Hz, 1H), 8.58 (dd, $J$ = 4.9, 1.5 Hz, 1H); MS (ESI, pos.) *m*/*z*: 540 (M+1). Compound **10** (less polar): *N*-{5-[1-(4-*tert*-butylphenyl)-3-pyridin-3-yl-1*H*-pyrazol-5-yl]pentanoyl}-L-tyrosinamide trifluoroacetate salt, $^1$H NMR (400 MHz, DMSO): $\delta$ = 1.34 (s, 9H), 1.47 (m, 4H), 2.05 (t, $J$ = 9.1 Hz, 2H), 2.61 (m, 1H), 2.64 (t, $J$ = 6.9 Hz, 2H), 2.84 (m, 1H), 4.34 (m, 1H), 6.30 (br s, 1H), 6.59 (d, $J$ = 8.4 Hz, 2H), 6.98 (m, 1H), 6.99 (d, $J$ = 8.4 Hz, 2H), 7.02 (s, 1H), 7.37 (m, 1H), 7.48 (d, $J$ = 8.8 Hz, 2H), 7.58 (d, $J$ = 8.8 Hz, 2H), 7.86 (d, $J$ = 8.4 Hz, 1H), 7.92 (dd, $J$ = 8.1, 5.5 Hz, 1H), 8.72 (dt, $J$ = 8.4, 1.5 Hz, 1H), 8.76 (dd, $J$ = 5.5, 1.5 Hz, 1H), 9.24 (d, $J$ = 2.2 Hz, 1H); MS (ESI, pos.) *m*/*z*: 540 (M+1); $[\alpha]_D^2$ +8.9 (*c* 0.51, MeOH).

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16. Compounds with purity lower than 60% by LC/MS were not considered.
17. Production cAMP in response to LH or small molecules was measured in CHO cells stably transfected with h-LH receptor. The cells were plated at a density of 20,000 cells/wells in growth medium (MEMalpha plus, 10% FBS, 1% L-glutamine, and 600 μg/ml Geneticin) in 96-well plates 1 day prior to the assay. Stimulation was carried out in assay buffer (phenol red-free DMEM/F12 containing 0.1% BSA, 0.1 mmol/L IBMX, and 1% pen-strep) for 60 min with increasing doses of test molecules. Following stimulation, cells were lysed and cAMP in the lysate was measured using a cAMP chemiluminescent assay kit (Tropix, Bedford, MA, USA) as per manufacturer's instructions.
18. LH-stimulated testosterone production or small molecule LH-mimetic activity was measured in Leydig cells harvested from male Sprague–Dawley rats between the ages of 21 and 40 days. Decapsulated testis was digested in collagenase-containing media (phenol red-free DMEM/F12 + 0.1% collagenase) at 37 °C in 5% $CO_2$ for 15 min. Cells were washed, plated at a density of 350,000 cells/well in 96-well plates (in phenol red-free DMEM/F12 media with 0.1% BSA), and stimulated with test compounds for 3 h. The plate was centrifuged to bring down the cells and 50 μL of culture media transferred to an ELISA plate (DSL) for testosterone measurement.
19. In vivo testosterone induction activity assay: on day 1, adult Sprague–Dawley rats were implanted subcutaneously in the scalpular region with a single 7-day slow-release pellet containing 7.5 mg of diethylstilbestrol (DES). After two days, rats were injected intraperitoneally with compound **10** at 0, 4, 16, or 32 mg/kg. Three rats were used in each treatment group. Blood samples were removed from the rat by retro-orbital plexus disruption after two hours following the administration. Serum was collected from blood by centrifugation and frozen at −80 °C until the time of assay. Serum testosterone concentrations and standard deviations were determined by ELISA and analyzed using Microsoft Excel software program.