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Development of Dipeptidic *h*GPR54 Agonists

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A series of dipeptides were designed as potential agonists of the human KiSS1-derived peptide receptor (*h*GPR54). While the sequence Arg-Trp-NH_2 was the most efficient in terms of affinity, we established a convergent synthetic strategy to optimize the N terminus. Using two successive Sonogashira cross-coupling reactions on a solid-supported peptide, we were able to introduce various alkynes at the N terminus to afford compounds with sub-micromolar affinities for *h*GPR54. However, functional assays indicated the benzoylated dipeptide Bz-Arg-Trp-NH_2 as the most promising compound in terms of agonistic properties. Interestingly, this compound appeared much more stable than the endogenous neuropeptide kisspeptin, both in serum and in liver microsomes of rats. This compound was also found to be able to induce a significant in vivo increase in testosterone levels in male rats.

Introduction

GPR54, also known as the KiSS1-derived peptide receptor (or KiSS1R), belongs to the G-protein-coupled receptor (GPCR) superfamily and is coupled to Gq/11 GTP binding protein.[1] *h*GPR54 is a member of the GPCR subfamily called the RF-amide receptors, which also includes NPFF1R, NPFF2R, GPR10, and GPR103. This subfamily of GPCR is known to bind endogenous RF-amide neuropeptides that share the C-terminal sequence Arg-Phe-NH_2. In 2001, three independent research groups identified kisspeptin,[2] a 54-residue peptide, as the endogenous ligand for GPR54, a known suppressor of metastasis.[3] Kisspeptin was originally named metastin for its ability to inhibit metastasis, but was later identified as a potent stimulator of gonadotropin-releasing hormone (GnRH) release, and thus critical for regulation of reproduction.[4] Expressed in several parts of the brain (hypothalamus, hippocampus, amygdala, etc.),[5] GPR54 and its endogenous ligand kisspeptin were also localized in dorsal root ganglia (DRG) neurons, and are involved in the regulation of pain sensitivity in rodents.[6] Recently, pharmacological experiments have investigated the role of various forms of kisspeptin in pubertal development and adult reproduction. However, kisspeptin-10 (Kp10) shows a poor metabolic stability, due to the cleavage of the Gly–Leu peptide bond by matrix metalloproteinases (MMPs).[7] To circumvent this metabolic instability, several Kp10 analogues were reported with modifications of the peptidic sequence including unnatural D-amino acids and peptidomimetic bonds.[7,8] We recently published a study about the effects of N-terminal deletions of endogenous RF-amide peptides on their affinity for NPFF1R, NPFF2R, GPR10, GPR54, and GPR103.[9] We showed that systematic and sequential N-terminal deletions of Kp10 decrease the affinity for *h*GPR54, but do not affect the agonistic character. Even the smaller dipeptide (Bz-RF-NH_2) was found to be a full agonist in the micromolar range ($pEC_{50} = 6.40$, $E_{max} = 100\,\%$).[9b] Several acylated RF-NH_2 have been previously reported as antagonists of NPFF receptors, and they were stable enough to show potent in vivo efficacies at low doses on several animal models after intravenous administration.[10] Starting from Bz-RF-NH_2, we propose herein a structure–activity relationship study of this dipeptide, using an original solid-phase synthesis based on sequential palladium-catalyzed reactions.

Results and Discussion

Previous works have shown that C-terminal phenylalanine may efficiently be replaced by tryptophan[11] or tyrosine.[8a] Using a classical Fmoc strategy on solid phase, we performed a scan of the C-terminal position, replacing the phenylalanine residue with other amino acids (**1**–**12**, Table 1). Only L-tryptophan led to a significant gain of affinity (**3**, $K_i = 974$ nM), whereas all other substitutions resulted in a loss of affinity. Next, we performed an exploration of the vicinity of the benzamide moiety at the N terminus (Table 2, **13**–**19**), but all the substitutions led also to a decrease in affinity. Alanine scans and N-terminal deletion studies performed on the Kp10 sequence have already shown the importance of the phenylalanine residue at position 5,[10a,12] highlighting the potential presence of a hydrophobic pocket in the GPR54 binding site. In an attempt to explore

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Table 1. Exploration of the C terminus of Bz-RF-NH_2.

Compd	Sequence	*h*GPR54 K_i [nM][a] or Inh. [%][b]
Kp10	H-YNWNSFGLRF-NH_2	0.5 ± 0.1
1	Bz-Arg-Phe-NH_2	7932 ± 857
2[c]	Bz-Arg-Hph-NH_2	0%
3	Bz-Arg-Trp-NH_2	974 ± 259
4	Bz-^{D}Arg-Trp-NH_2	0%
5	Bz-Arg-^{D}Trp-NH_2	3%
6	Bz-^{D}Arg-^{D}Trp-NH_2	2%
7	Bz-Arg-Trp(Me)-NH_2	4%
8	Bz-Arg-Tyr-NH_2	23%
9	Bz-Arg-His-NH_2	0%
10	Bz-Arg-Leu-NH_2	17%
11	Bz-Arg-Asn-NH_2	2%
12	Bz-Arg-Gln-NH_2	0%

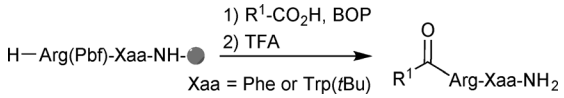
[a] [^{3}H]Kp13 was used as radioligand for competition assays; values are the mean ± SEM of at least two independent determinations performed in duplicate. [b] Percent inhibition at 5 μM. [c] Hph = homophenylalanine.

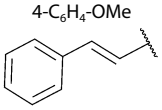
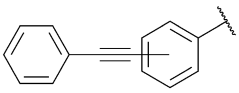
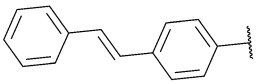
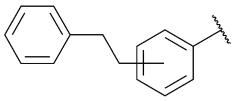
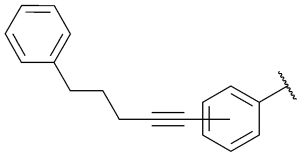
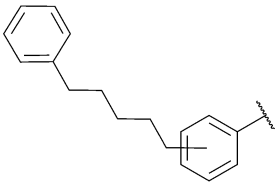
this additional lipophilic pocket, we grafted an aromatic ring to the benzamide moiety at various positions using spacers (Table 2, **20**–**33**). The *para* position appeared more favorable than the *meta* position, and the best compounds were those with a rigid spacer, such as a phenylacetylene group (**26**, K_i = 326 nM) or a styryl group (**27**, K_i = 322 nM). Interestingly, the more flexible **29** with a phenethyl group displayed lower affinity (K_i = 5.2 μM) than **25** with a rigid spacer (K_i = 1.555 μM). As observed previously (Table 1), tryptophan at the C terminus led to a significant improvement over phenylalanine.

Following a classical optimization process, we next introduced chemical diversity on the phenylacetylene moiety. All the compounds listed in Table 2 were prepared by following a standard solid-phase strategy, in which all the N-terminal moieties were prepared separately as carboxylic acids (see the Supporting Information). As the introduction of chemical diversity around the phenylacetylene group would require many carboxylic acids to prepare as precursors, we developed an innovative and convergent chemical strategy aimed at introducing chemical diversity through palladium-catalyzed cross-coupling reactions performed directly on the dipeptide supported on resin (Scheme 1). Palladium-catalyzed cross-coupling reactions (CCR) have already been applied to solid-phase synthesis,[13] including Suzuki–Miyaura, Heck, Negishi, Sonogashira, and Buchwald–Hartwig. However, these reactions were mainly applied on supported heterocycles, compatible with high temperatures and strong bases. In our case, the challenge was to perform racemization-free Sonogashira CCR on a supported peptide. To improve the optimization of compound **26**, we chose to perform two successive Sonogashira CCRs (Scheme 1). Starting from the supported *para*-iodobenzamide **34**, a first Sonogashira CCR was performed with trimethylsilylacetylene under standard conditions: $PdCl_2(PPh_3)_2$/CuI in the presence of Et_3N in DMF at room temperature. To characterize the cross-coupling reaction, the resin, as well as TMS and *t*Bu moieties, were cleaved with TFA to afford the terminal alkyne **38** with a purity of 95% at 210 nm (HPLC). After purification by chromatography, **38** was obtained in 65% overall yield. After this encouraging initial Sonogashira CCR, the trimethylsilyl protecting group of **36** was selectively removed with TBAF in THF at room temperature to afford the corresponding supported terminal alkyne, which was directly engaged in the second Sonogashira CCR using phenylbromide or phenyliodide under the same conditions previously used. Surprisingly, **26** was obtained in only 13% yield with phenyliodide, whereas no reaction was observed with phenylbromide (Table 3, entries 1 and 2). Therefore, we decided to undertake the chemical optimization of this second Sonogashira CCR (Table 3). Cerezo et al. previously showed that Suzuki–Miyaura CCR on supported peptides is feasible using microwave (MW) heating for a short time (t = 10 min).[14] Indeed, starting from supported Arg-Phe derivative **36**, and using MW heating at 80 °C for only 2 min, we were able to obtain **25** in 13% yield (Table 3, entry 3). This result was modest, but still remarkable as we used phenylbromide as reagent. Instead of increasing the heating conditions (temperature or time), we choose to screen a series of palladium catalysts (Table 3, entries 3–8). All of them afforded **25** in yields ranging from 8 to 18%, similar to $Pd(PPh_3)_2Cl_2$ (13% yield), except for $Pd(P(tBu)_3)_2$, which led to a promising 42% yield. However, $Pd(P(tBu)_3)_2$ is difficult to handle, as it is known to be air sensitive, as is the tri-*tert*-butylphosphine $P(tBu)_3$. Consequently, we used the air-stable tributylphosphonium salt described by Netherton and Fu as an in situ precursor of $P(tBu)_3$.[15] We screened a series of five palladium catalysts in presence of $P(tBu)_3{\cdot}HBF_4$, and all of them led to yields ranging from 30 to 39% (Table 3, entries 9–13). Whereas heating is crucial to initiate the reaction (entry 15), an increase in heating time up to 20 min did not improve yield (entry 14). Phenyliodide was a bit more reactive than phenylbromide, and almost no reaction occurred with phenylchloride. Although the yield was still modest (~40% overall yield), compound **25** was recovered up to 88% purity (HPLC) after a simple trituration in Et_2O. Moreover, we compared the resulting compound **25** with that previously obtained by directly coupling Ph-≡-C_6H_4-COOH to the supported dipeptide Arg-Phe-NH-Rink resin. HPLC and ^{1}H NMR were identical, and no racemization was detected. Based on this efficient procedure, we synthesized a series of derivatives of compound **26** (Table 4, **38**–**46**). In this series based on the Arg-Trp scaffold, overall yields were higher than that obtained with the sequence Arg-Phe, ranging from 38 to 85%. In terms of affinity, all of them exhibited affinities in the micromolar range for *h*GPR54, showing that the phenylacetylene moiety does not tolerate any substitution. Based on their affinities, three compounds (**3**, **26**, and **27**) were selected for further evaluation of their agonistic properties, and also for their metabolic stability (Table 5). We compared them with the pentapeptide **47** (Bz-FGLRF-NH_2), which has a phenylalanine residue at the N terminus, crucial for affinity,[8c] but also the sequence Phe-Gly-Leu-Arg, known to be hydrolyzed in serum.[7]

Interestingly, the dipeptide **3** appeared to be a potent full agonist of *h*GPR54 (EC_{50} = 69 nM). In contrast, the introduction of phenylacetylene (**26**) or styryl (**27**) moieties led to more rigid molecules, improving affinity, but altering agonistic prop-

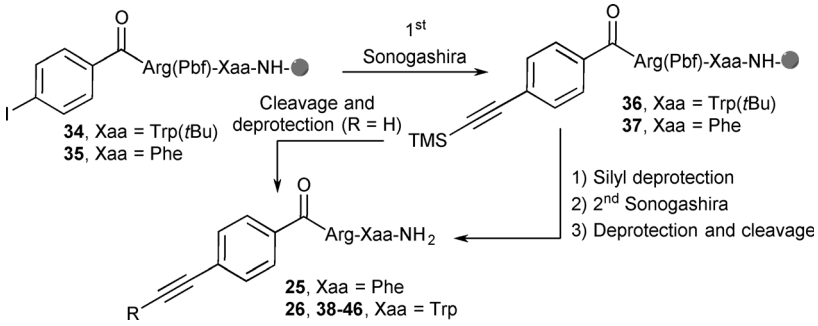
Table 2. Exploration of the N termini of Bz-RF-NH_2 (**1**) or Bz-RW-NH_2 (**3**).



Compd	$R^1C(O)$-Arg-Xaa-NH_2 R^1	Xaa	*h*GPR54[a] Inh. [%][b]	*h*GPR54[a] K_i [nM][c]
1	Ph	Phe	66	7932 ± 857
3		Trp	100	974 ± 259
13	2-C_6H_4-Cl	Phe	40	> 10 μM
14	3-C_6H_4-Cl	Phe	39	> 10 μM
15	4-C_6H_4-Cl	Phe	27	> 10 μM
16	2-C_6H_4-OMe	Phe	29	> 10 μM
17	3-C_6H_4-OMe	Phe	44	> 10 μM
18	4-C_6H_4-OMe	Phe	17	> 10 μM
19		Phe	35	> 10 μM
20	2-C_6H_4-Ph	Phe	21	> 10 μM
21	3-C_6H_4-Ph	Phe	24	> 10 μM
22	4-C_6H_4-Ph	Phe	70	4872 ± 1489
23		Trp	100	747 ± 95
24	*meta* 	Phe	43	> 10 μM
25	*para*	Phe	92	1555 ± 348
26	*para*	Trp	100	326 ± 28
27	*para* 	Trp	100	322 ± 91
28	*meta* 	Phe	32	> 10 μM
29	*para*	Phe	62	5208 ± 608
30	*meta* 	Phe	34	> 10 μM
31	*para*	Phe	89	3135 ± 345
32	*meta* 	Phe	23	> 10 μM
33	*para*	Phe	60	> 10 μM

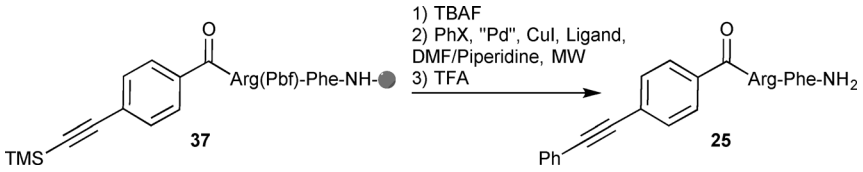
[a] [^{3}H]Kp13 was used as radioligand for competition assays. [b] Percent inhibition at 5 μM. [c] Values are the mean ± SEM of at least two independent determinations performed in duplicate.

erties. Still, **26** and **27** are full agonists of *h*GPR54 in the sub-micromolar range. Next, we investigated the metabolic stability of these compounds (Table 5). Whereas the pentapeptide **47** showed poor stability both in serum and in liver microsomes, dipeptides **3**, **26**, and **27** appeared much more stable under both conditions. As peripheral administration of kisspeptin is known to increase circulating luteinizing hormone (LH) and testosterone,[16] peptides **3**, **26** and **27** were tested for their ability to increase the production of testosterone in comparison with kisspeptin (Figure 1). Compounds were tested at 10 mg kg^{-1} (i.p.) in male rats, but **26** appeared poorly soluble in the vehicle (ethanol/Ringer [1:1]), and was not tested. Despite acting as full agonist at GPR54 in the sub-micromolar range, **27** was not potent enough to stimulate testosterone.



Scheme 1. Supported synthetic strategy leading to compounds **25**, **26**, and **38**–**46**.

Table 3. Optimization of the second Sonogashira CCR.



Entry	PhX	Pd source	Ligand	MW[a] T [°C]	MW[a] t [min]	Yield [%][b]
1	PhI	$Pd(PPh_3)_2Cl_2$	–	RT		13[c]
2	PhBr	$Pd(PPh_3)_2Cl_2$	–	RT		0
3	PhBr	$Pd(PPh_3)_2Cl_2$	–	80	2	13
4	PhBr	$Pd(PPh_3)_4$	–	80	2	18
5	PhBr	$Pd(dppf)Cl_2$	–	80	2	8
6	PhBr	$Pd(P(tBu)_3)_2$	–	80	2	42
7	PhBr	$Pd(MeCN)_2Cl_2$	–	80	2	15
8	PhBr	$Pd(PhCN)_2Cl_2$	–	80	2	16
9	PhBr	$Pd(OAc)_2$	$P(tBu)_3{\cdot}HBF_4$	80	2	30
10	PhBr	$Pd(PPh_3)_2Cl_2$	$P(tBu)_3{\cdot}HBF_4$	80	2	39
11	PhBr	$Pd(P(tBu)_3)_2$	$P(tBu)_3{\cdot}HBF_4$	80	2	28
12	PhBr	$Pd(MeCN)_2Cl_2$	$P(tBu)_3{\cdot}HBF_4$	80	2	33
13	PhBr	$Pd(PhCN)_2Cl_2$	$P(tBu)_3{\cdot}HBF_4$	80	2	31
14	PhBr	$Pd(PhCN)_2Cl_2$	$P(tBu)_3{\cdot}HBF_4$	80	20	34
15	PhBr	$Pd(PhCN)_2Cl_2$	$P(tBu)_3{\cdot}HBF_4$	RT		0
16	PhI	$Pd(PhCN)_2Cl_2$	$P(tBu)_3{\cdot}HBF_4$	80	2	43
17	PhCl	$Pd(PhCN)_2Cl_2$	$P(tBu)_3{\cdot}HBF_4$	80	2	1

[a] Microwave. [b] Yield over nine steps determined by HPLC/UV using caffeine as external standard. [c] Yield after 16 h.

Conversely, the small benzoylated dipeptide **3** led to a significant increase in testosterone, similar to that induced by kisspeptin. This result is particularly interesting if we consider the metabolic stability of **3** in comparison with kisspeptin. As the Arg-Phe-NH_2 sequence is known to bind efficiently to both NPFF1 and 2 receptors, we tested **3** on both receptors, and we found micromolar affinities (NPFF1R: $K_i = 2.4$ μM, NPFF2R: $K_i = 1.0$ μM) in the same range of its affinity for *h*GPR54.

Conclusions

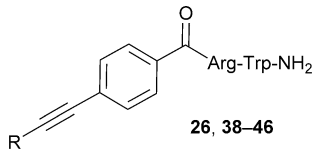
Using a rational design along with metal-catalyzed reactions on supported dipeptides, we were able to develop promising ligands (**26** and **27**) of *h*GPR54. However, these compounds appeared to be less potent agonists than the simple benzoylated dipeptide Bz-Arg-Trp-NH_2 (**3**). This small dipeptide appeared to be much more stable than the endogenous peptide kisspeptin, and although exhibiting a lower EC_{50} value (69 nM) than kisspeptin (< 1 nM), it clearly induced a significant increase in levels of circulating testosterone when administered to rats at a dose of 10 mg kg^{-1} (i.p.). In conclusion, **3** is a promising compound for optimization, and more synthetic work will be required to improve its selectivity toward other RFamide receptors such as NPFF1R and NPFF2R.

Experimental Section

Chemistry

Materials: Chemicals and solvents were used without further purification. Compounds were purified using Armen spot flash chromatography on silica gel Merck 60 (particle size 0.040–0.063 mm), or on simply connect C_{18} from AIT. Yields refer to isolated compounds, with purity > 95% as determined by HPLC. ^{1}H and ^{13}C NMR spectra were recorded at 400/500 MHz and 101/128 MHz,

Table 4. Yields obtained for **26**, **38–46**, and their affinities for *h*GPR54.



26, **38–46**

Compd	R	Yield [%][a]	*h*GPR54 K_i [nM][b]
26	Ph		326 ± 28
38	H	65	> 10 μM
39	2-C_6H_4-Cl	75	> 10 μM
40	3-C_6H_4-Cl	85	5290 ± 1398
41	4-C_6H_4-Cl	39	7122 ± 2764
42	3-C_6H_4-OMe	42	2181 ± 685
43	4-C_6H_4-OMe	75	> 10 μM
44	2-pyridinyl	38	> 10 μM
45	3-pyridinyl	73	2891 ± 496
46	4-pyridinyl	52	2897 ± 492

[a] Isolated yield over nine steps. [b] [^{3}H]Kp13 was used as radioligand for competition assays; values are the mean ± SEM of at least two independent determinations performed in duplicate.

Table 5. Functional activity toward *h*GPR54 and metabolic stability.

	Compd	EC_{50} [nM][a]	Stability	
			Rat serum $t_{1/2}$ [min]	CL_{int} [b]
47	Bz-Phe-Gly-Leu-Arg-Phe-NH_2	0.55 ± 0.06	32 ± 4	36 ± 5
3	Bz-Arg-Trp-NH_2	69 ± 1.8	121 ± 28	< 3
26	Ph-≡-Bz-Arg-Trp-NH_2	700 ± 99	429 ± 8	< 3
27	Ph-═-Bz-Arg-Trp-NH_2	390 ± 48	196 ± 10	3.4 ± 3

[a] *h*GPR54 calcium mobilization assay: values are the mean ± SEM of at least two independent determinations performed in duplicate. [b] Clearance [μL min^{-1} (mg protein)$^{-1}$] determined in rat liver microsomes.

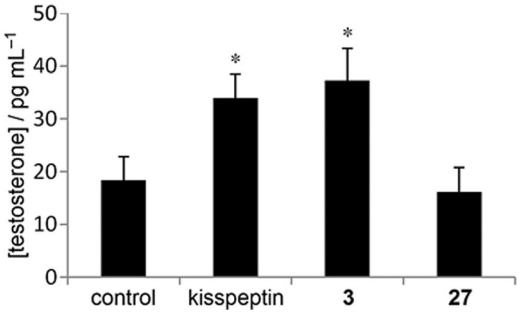


Figure 1. Effect of GPR54 agonists on circulating testosterone in adult male rats. Kisspeptin and compounds **3** and **27** were dissolved in ethanol/Ringer's solution (1:1) and injected intraperitoneally (10 mg kg^{-1}). Testosterone was measured by using a commercial radioimmunoassay kit from blood sampled 30 min post-injection. Values are the mean ± SEM of $n = 6$ rats per experimental point; statistical significance: $^*p < 0.05$ relative to control.

respectively. All chemical shift values (δ) and coupling constants (J) are quoted in ppm and in Hz, respectively. Microwave irradiations were performed using Biotage Initiator EXP.

General solid-phase peptide synthesis procedures: All peptides were synthesized by manual solid-phase synthesis by using an Fmoc strategy with a Rink-amide resin SS (Advanced ChemTech, 0.75 mmol g^{-1}). The side-chain protective groups included *tert*-butyl (*t*Bu) for tyrosine, *tert*-butoxycarbonyl (Boc) for tryptophane, trityl (Trt) for histidine, asparagine, and glutamine, and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for arginine. Fmoc deprotection was performed by using a solution of 2% piperidine and 2% DBU in DMF. Coupling reactions were performed by the addition of *N*,*N*-diisopropylethylamine (DIEA, 4.5 equiv), Fmoc-protected amino acid or carboxylic acid (3 equiv), and (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP, 2.85 equiv) in CH_2Cl_2, and the mixture was agitated for 1 h. All coupling reactions were monitored by Kaiser test. Resin cleavage was performed by using a solution of TFA/thioanisole/triisopropylsilane (92:5:3). The mixture was filtered, and the peptide solution was concentrated in vacuo, and precipitated by adding cold Et_2O. The mixture was centrifuged, and the resulting crude peptide was purified by flash chromatography (Simply connect C_{18} column (50 g, 40–60 μm) from AIT. Phase A: 0.05% TFA in H_2O, phase B: MeOH; eluent: 10→100% phase B in 30 min. Final compounds **1**–**33** and **47** were recovered with purity > 95% as determined by HPLC (see the Supporting Information).

***N*-(benzoyl)-Arg-Trp-NH_2, trifluoroacetate salt 3**: ^{1}H NMR (500 MHz, CD_3OD): $\delta = 7.79$–7.72 (m, 2H), 7.61–7.59 (m, 1H), 7.58–7.52 (m, 1H), 7.50–7.40 (m, 2H), 7.31–7.23 (m, 1H), 7.12 (s, 1H), 7.08–7.02 (m, 1H), 7.0–6.95 (m, 1H), 4,72 (dd, $J = 5.8$, 8.0 Hz, 2H), 4.52 (dd, $J = 6.3$, 8.2 Hz, 1H), 3.23–3.14 (m, 4H), 1.91–1.80 (m, 1H), 1.80–1.73 (m, 1H), 1.56 ppm (s, 2H); ^{13}C NMR (126 MHz, CD_3OD): $\delta = 176.9$, 174.0, 170.9, 158.9, 138.3, 135.3, 133.3, 129.9, 129.1, 128.9, 125.0, 122.8, 120.2, 119.6, 112.6, 111.1, 55.4, 55.2, 42.2, 30.1, 29.2, 26.5 ppm; MS (MM-ES-APCI) m/z $[M+H]^+$ calcd for $C_{24}H_{29}N_7O_3$: 464.2, found 464.2.

General procedure to introduce trimethylsililylacetylene on solid-phase by Sonogashira reaction (35–36): Supported *N*-(4-iodobenzoyl)-L-Arg-Xaa-NH-resin (Xaa = L-Phe or L-Trp(*t*Bu)) were synthesized using the general solid-phase peptide synthesis procedures. In a syringe with frit, resin was suspended in anhydrous DMF ($c = 0.1$ mmol mL^{-1}), followed by the addition of trimethylamine (20 equiv) and trimethylsilylacetylene (5 equiv). Reaction mixture was degassed with nitrogen, before adding $PdCl_2(PPh_3)_2$ (0.2 equiv) and CuI (0.4 equiv). The reaction was stirred overnight with an orbital mixer at room temperature. Resin was then washed successively with DMF, CH_2Cl_2, MeOH, and Et_2O.

General procedure to trimethylsilyl deprotection and second Sonogashira coupling reaction on solid phase (25–26, 38–46): TBAF (1 M in THF) was introduced in a syringe containing the supported dipeptide **36** or **37**, and stirred for 3 h at room temperature. Solid phase was then washed successively with THF, DMF, CH_2Cl_2, MeOH, and Et_2O. Resin was transferred to a microwave reactor, and a mixture of anhydrous DMF/piperidine [*v*/*v*] ($c = 0.28$ mmol mL^{-1}) was added, followed by haloaryl or heteroaryl (5 equiv). Reaction mixture was degassed with nitrogen, and $PdCl_2(PhCN)_2$ (0.05 equiv), $PtBu_3{\cdot}HBF_4$ (0.1 equiv) and CuI (0.1 equiv) were introduced. The microwave tube was then capped properly and the reaction mixture was stirred for 2 to 20 min at 80 °C under microwave irradiation. Resin was then transferred to a syringe with frit, and was washed successively with DMF, CH_2Cl_2, MeOH, Et_2O. Cleavage of the pep-

tide–resin complexes was performed by treatment with trifluoroacetic acid/triisopropylsilane/thioanisole (92:3:5, *v/v/v*) at room temperature for 2 h. After filtration of the resin, anhydrous Et_2O at 4 °C was added to the soluble material to cause precipitation of the crude peptide, which was collected as a pellet by centrifugation. Purification was performed as described previously for classical peptides, and final compounds (**25**–**26**, **38**–**46**) were recovered with purity > 95% by HPLC.

***N*-(4-phenylethynylbenzoyl)-Arg-Phe-NH$_2$, trifluoroacetate salt 25**: ^{1}H NMR (500 MHz, CD_3OD): δ = 7.83–7.86 (m, 2H), 7.60–7.63 (m, 2H), 7.52–7.56 (m, 2H), 7.38–7.42 (m, 3H), 7.21–7.26 (m, 2H), 7.16–7.21 (m, 2H), 7.11–7.16 (m, 1H), 4.66 (dd, *J* = 5.36, 9.14 Hz, 1H), 4.53 (dd, *J* = 6.3, 8.2 Hz, 1H), 3.13–3.23 (m, 3H), 2.87–2.99 (m, 1H), 1.82–1.92 (m, 1H), 1.71–1.82 (m, 1H), 1.52–1.64 ppm (m, 2H); ^{13}C NMR (126 MHz, CD_3OD): δ = 176.4, 174.1, 170.1, 159.1, 138.2, 134.4, 133.2, 133.0, 130.8, 130.4, 130.1, 129.9, 129.3, 128.8, 128.3, 124.5, 93.2, 89.7, 56.1, 55.4, 42.4, 39.3, 30.2, 26.7 ppm; MS (MM-ES-APCI) *m/z* $[M+H]^+$ calcd for $C_{30}H_{32}N_6O_3$: 525.2, found 525.2.

***N*-(4-ethynylbenzoyl)-Arg-Trp-NH$_2$, trifluoroacetate salt 38**: ^{1}H NMR (400 MHz, CD_3OD): δ = 7.75 (d, *J* = 8.3 Hz, 2H), 7.60 (d, *J* = 8.0 Hz, 1H), 7.53 (d, *J* = 8.3 Hz, 2H), 7.28 (d, *J* = 8.0 Hz, 1H), 7.12 (s, 1H), 7.08–7.01 (m, 1H), 7.01–6.91 (m, 1H), 4.71 (dd, *J* = 5.5, 8.3 Hz, 1H), 4.45 (dd, *J* = 6.5, 8.0 Hz, 1H), 3.69 (s, 1H), 3.26–3,17 (m, 2H), 3.17–3.06 (m, 2H), 1.87–1.72 (m, 2H), 1.60–1.45 ppm (m, 2H); ^{13}C NMR (101 MHz, CD_3OD): δ = 176.3, 174.2, 169.9, 159.3, 138.3, 135.1, 133.4, 129.1, 127.7, 125.1, 122.8, 120.2, 119.7, 119.6, 116.9, 111.0, 83.9, 81.6, 55,8, 55.6, 42.2, 29.8, 29.0, 26.6 ppm; MS (MM-ES-APCI) *m/z* $[M+H]^+$ calcd for $C_{26}H_{29}N_7O_3$: 488.2, found 488.2.

***N*-(4-(2-chlorophenyl)ethynylbenzoyl)-Arg-Trp-NH$_2$, trifluoroacetate salt 39**: ^{1}H NMR (400 MHz, CD_3OD): δ = 7.81 (d, *J* = 8.28 Hz, 2H), 7.66–7.51 (m, 4H), 7.53–7.47 (m, 1H), 7.43–7.31 (m, 2H), 7.29 (d, *J* = 8.03 Hz, 1H), 7.13 (s, 1H), 7.10–7.02 (m, 1H), 7.02–6.92 (m, 1H), 4.72 (dd, *J* = 5.65, 8.41 Hz, 1H), 4.45 (dd, *J* = 6.53, 8.03 Hz, 1H), 3.27–3.15 (m, 2H), 3.13 (t, *J* = 7.15 Hz, 2H), 1.87–1.76 (m, 2H), 1.61–1.45 ppm (m, 2H); ^{13}C NMR (101 MHz, CD_3OD): δ = 177.0, 174.0, 170.0, 158.7, 138.1, 137.0, 134.7, 134.6, 132.7, 131.4, 130.6, 129.1, 128.2, 127.8, 124.9, 122.6, 120.0, 119.5, 119.4, 116.6, 112.5, 110.8, 94.5, 89.4, 55.7, 55.4, 42.0, 29.6, 28.7, 26.4 ppm; MS (MM-ES-APCI) *m/z* $[M+H]^+$ calcd for $C_{32}H_{32}ClN_7O_3$: 598.2, found 598.2.

***N*-(4-(3-chlorophenyl)ethynylbenzoyl)-Arg-Trp-NH$_2$, trifluoroacetate salt 40**: ^{1}H NMR (400 MHz, CD_3OD): δ = 7.79 (d, *J* = 8.3 Hz, 2H), 7.61–7.59 (m, 3H), 7.55 (s, 1H), 7.49–7.46 (m, 1H), 7.44–7.37 (m, 2H), 7.30 (d, *J* = 8.0 Hz, 1H), 7,14 (s, 1H), 7.08–7.01 (m, 1H), 7.01–6.92 (m, 1H), 4.71 (dd, *J* = 5.5, 8.3 Hz, 1H), 4.45 (dd, *J* = 7.0, 7.5 Hz, 1H), 3.25–3.16 (m, 2H), 3.13 (t, *J* = 7.2 Hz, 2H), 1.90–1.71 (m, 2H), 1.63–1.47 ppm (m, 2H); ^{13}C NMR (101 MHz, CD_3OD): δ = 176.8, 174.0, 170.0, 158.4, 138.0, 135.5, 134.7, 132.8, 132.3, 131.4, 131.2, 130.2, 129.0, 127.6, 125.9, 124.9, 122.6, 120.1, 119.4, 116.6, 112.5, 110.7, 91.1, 90.6, 55.6, 55.3, 42.0, 40.4, 28.7, 26.8 ppm; MS (MM-ES-APCI) *m/z* $[M+H]^+$ calcd for $C_{32}H_{32}ClN_7O_3$: 598.2, found 598.2.

***N*-(4-(4-chlorophenyl)ethynylbenzoyl)-Arg-Trp-NH$_2$, trifluoroacetate salt 41**: ^{1}H NMR (400 MHz, CD_3OD): δ = 7.69 (d, *J* = 8.3 Hz, 2H), 7.54–7.48 (m, 3H), 7.44 (d, *J* = 8.5 Hz, 2H), 7.32 (d, *J* = 8.5 Hz, 2H), 7.20 (d, *J* = 8.0 Hz, 1H), 7.03 (s, 1H), 6.98–6.94 (m, 1H), 6.92–6.83 (m, 1H), 4.66–4.57 (m, 1H), 4.38 (dd, *J* = 6.4, 8.4 Hz, 1H), 3.14–3.06 (m, 2H), 3.04 (t, *J* = 7.2 Hz, 2H), 1.79–1.65 (m, 2H), 1.52–1.39 ppm (m, 2H); ^{13}C NMR (101 MHz, CD_3OD): δ = 176.8, 173.8, 170.0, 168.9, 138.2, 136.0, 134.6, 134.2, 132.6, 130.0, 128.9, 127.8, 124.7, 122.5, 119.9, 119.5, 119.3, 116.6, 112.4, 110.7, 94.6, 90.3, 55.3, 55.2, 41.9, 29.7, 28.7, 26.2 ppm; MS (MM-ES-APCI) *m/z* $[M+H]^+$ calcd for $C_{32}H_{32}ClN_7O_3$: 598.2, found 598.2.

***N*-(4-(3-methoxyphenyl)ethynylbenzoyl)-Arg-Trp-NH$_2$, trifluoroacetate salt 42**: ^{1}H NMR (400 MHz, CD_3OD): δ = 7.87–7.73 (m, 2H), 7.68–7.57 (m, 3H), 7.39–7.28 (m, 2H), 7.15 (s, 1H), 7.14–7.05 (m, 3H), 7.05–6.92 (m, 2H), 4.74 (dd, *J* = 5.5, 8.3 Hz, 1H), 4.49 (dd, *J* = 6.3, 8.0 Hz, 1H), 3.84 (s, 3H), 3.27–3.18 (m, 2H), 3.15 (t, *J* = 7.3 Hz, 2H), 1.92–1.76 (m, 2H), 1.62–1.50 ppm (m, 2H); ^{13}C NMR (101 MHz, CD_3OD): δ = 176.9, 173.8, 169,9, 161.1, 158.6, 138.0, 134.3, 132.6, 130.8, 128.9, 128.8, 128.2, 125.2, 125.0, 124.8, 122.5, 119.9, 119.3, 117.7, 116.3, 112.4, 110.7, 92.7, 89.1, 55.9, 55.4, 55.2, 41.9, 30.7, 26.2, 24.2 ppm; MS (MM-ES-APCI) *m/z* $[M+H]^+$ calcd for $C_{33}H_{35}N_7O_4$: 594.2, found 594.2.

***N*-(4-(4-methoxyphenyl)ethynylbenzoyl)-Arg-Trp-NH$_2$, trifluoroacetate salt 43**: ^{1}H NMR (400 MHz, CD_3OD): δ = 7.85–7.74 (m, 2H), 7.70 (d, *J* = 7.0 Hz, 1H), 7.65–7.55 (m, 2H), 7.31–7.21 (m, 3H), 7.11 (s, 1H), 7.05–6.88 (m, 4H), 4.69 (dd, *J* = 5.7, 8.7 Hz, 1H), 4.39 (dd, *J* = 7.2 Hz, 1H), 3.84 (s, 3H), 3.26–3.13 (m, 2H), 3.10 (t, *J* = 7.0 Hz, 2H), 1.87–1.70 (m, 2H), 1.58–1.40 ppm (m, 2H); ^{13}C NMR (101 MHz, CD_3OD): δ = 178.3, 173.9, 169.8, 164.0, 159.3, 136.5, 135.6, 133.4, 132.2, 131.6, 131.3, 129.1, 124.9, 122.5, 122.5, 119.5, 116.6, 116.5, 113.7, 112.4, 93.4, 87.6, 56.2, 55.7, 55.4, 42.0, 26.3, 24.7, 24.3 ppm; MS (MM-ES-APCI) *m/z* $[M+H]^+$ calcd for $C_{33}H_{35}N_7O_4$: 594.2, found 594.2.

***N*-(4-(2-pyridinyl)ethynylbenzoyl)-Arg-Trp-NH$_2$, trifluoroacetate salt 44**: ^{1}H NMR (400 MHz, CD_3OD): δ = 8.63–8.61 (m, 1H), 8.03–7.91 (m, 1H), 7.84 (d, *J* = 8.5 Hz, 2H), 7.75 (d, *J* = 7.8 Hz, 1H), 7.69 (d, *J* = 8.3 Hz, 2H), 7.61 (d, *J* = 7.8 Hz, 1H), 7.58–7.47 (m, 1H), 7.30 (d, *J* = 8.0 Hz, 1H), 7.15 (s, 1H), 7.11–7.01 (m, 1H), 7.01–6.90 (m, 1H), 4.73 (dd, *J* = 5.5, 8.3 Hz, 1H), 4.47 (dd, *J* = 6.9, 7,7 Hz, 1H), 3.24–3.18 (m, 2H), 3.14 (t, *J* = 7.0 Hz, 2H), 1.93–1.74 (m, 2H), 1.62–1.44 ppm (m, 2H); ^{13}C NMR (101 MHz, CD_3OD): δ = 177.4, 174.4, 170.3, 159.2, 151.0, 143.7, 139.8, 138.6, 135.8, 133.6, 129.7, 129.6, 127.2, 125.8, 125.3, 123.0, 122.8, 119.9, 117.0, 112.9, 111,2, 92.0, 90.5, 56.1, 55.8, 42.5, 30.0, 29.1, 26.8 ppm; MS (MM-ES-APCI) *m/z* $[M+H]^+$ calcd for $C_{31}H_{32}N_8O_3$: 565.2, found 565.2.

***N*-(4-(3-pyridinyl)ethynylbenzoyl)-Arg-Trp-NH$_2$, trifluoroacetate salt 45**: ^{1}H NMR (400 MHz, CD_3OD): δ = 8.86–8.75 (m, 1H), 8.65–8.53 (m, 1H), 8.24 (d, *J* = 8.0 Hz, 1H), 7.83 (d, *J* = 8,5 Hz, 2H), 7.74–7.68 (m, 1H), 7.66 (d, *J* = 8.3 Hz, 2H), 7.61 (d, *J* = 8.0 Hz, 1H), 7.29 (d, *J* = 8.0 Hz, 1H), 7.14 (s, 1H), 7.08–7.02 (m, 1H), 7.02–6.93 (m, 1H), 4.76–4.67 (m, 1H), 4.46 (dd, *J* = 7.2 Hz, 1H), 3.37 −3.32 (m, 1H), 3.18–3.22 (m, 1H), 3.13 (t, *J* = 7.2 Hz, 2H), 1.90–1.74 (m, 2H), 1.61–1.46 ppm (m, 2H); ^{13}C NMR (101 MHz, CD_3OD): δ = 176.7, 173.8, 169.7, 158.5, 150.5, 142.9, 137.9, 135.1, 132.8(2C), 129.0, 128.9, 128.6, 126.7, 124.7, 122.4, 122.1, 119.8, 119.2, 116.3, 112.3, 110.6, 93.9, 91.0, 55.4, 55.2, 41.8, 30.6, 29.4, 26.1 ppm; MS (MM-ES-APCI) *m/z* $[M+H]^+$ calcd for $C_{31}H_{32}N_8O_3$: 565.2, found 565.2.

***N*-(4-(4-pyridinyl)ethynylbenzoyl)-Arg-Trp-NH$_2$, trifluoroacetate salt 46**: ^{1}H NMR (400 MHz, CD_3OD): δ = 8.88–8.71 (m, 2H), 8.01–7.95 (m, 2H), 7.88–7.81 (d, *J* = 8.5 Hz, 2H), 7.77–7.66 (d, *J* = 8.5 Hz, 2H), 7.61 (d, *J* = 8.0 Hz, 1H), 7.29 (d, *J* = 8.0 Hz, 1H), 7.14 (s, 1H), 7.09–7.00 (m, 1H), 7.00–6.93 (m, 1H), 4.72 (dd, *J* = 5.5, 8.3 Hz, 1H), 4.47 (dd, *J* = 6.2, 7.9 Hz, 1H), 3.21–3.15 (m, 2H), 3.14 (t, *J* = 7.0 Hz, 2H), 1.89–1.74 (m, 2H), 1.58–1.56 ppm (m, 2H); ^{13}C NMR (101 MHz, CD_3OD): δ = 176.8, 173.8, 169.6, 158.7, 146.2, 146.0, 138.0, 136.2, 133.4, 129.2, 128.8, 125.7, 124.8, 122.5, 119.9, 119.4, 116.5, 112.4, 110.7, 88.5, 88.3, 55.5, 55.3, 41.9, 29.5, 26.3, 24.2 ppm; MS (MM-ES-APCI) *m/z* $[M+H]^+$ calcd for $C_{31}H_{32}N_8O_3$: 565.2, found 565.2

Biological methods

Materials: Probenecid was from Sigma–Aldrich (Saint Quentin Fallavier, France) and Fluo-4 acetoxymethyl (AM) ester from Molecular Probes (Invitrogen, Cergy Pontoise, France). Kp-10 and NPFF were from Polypeptide (Strasbourg, France). RFRP-3 was from Tebu-Bio (Le Perray-en-Yvelines, France). [^{3}H]FFRF-amide (13.6 Ci mmol^{-1}) was from the CEA (Saclay, France). [^{3}H]Kp13 (90 Ci mmol^{-1}) was from Hartmann Analytic (Braunschweig, Germany).

Adult male NMRI mice (Taconic, Ll Skensved) weighing 30–32 g were housed five animals per cage. The animals were kept under 12 h/12 h light/dark cycle, in a temperature-controlled room, with free access to food and water and acclimatized in the same cage for at least seven days before the experiment. All experiments were conducted in accordance with the Declaration of Helsinki, the Danish National Guide for Care and Use of Laboratory Animals, and the European Communities Council Directive (86/609/EEC; license number 2005/561–962).

Radioligand binding assays: Binding assay conditions were essentially as described previously.[9a] Briefly, membranes from CHO cells stably expressing *h*GPR54 were incubated with 0.15 nM [^{3}H]Kp13. Nonspecific binding levels were determined in the presence of 1 μM Kp10. NPFF1R- or NPFF2R-containing membranes were incubated with 10 or 3 nM [^{3}H]FFRF-NH_2, respectively. Nonspecific [^{3}H]FFRF-NH_2 binding levels were determined in the presence of 1 μM RFRP-3 (NPFF1R) or 1 μM NPFF (NPFF2R). Competition-type experiments were performed at 25 °C, under binding equilibrium conditions (1 h, 0.25 mL final volume) in the presence of increasing concentrations of unlabeled peptides or compounds to be tested for their binding affinity. Membrane-bound radioactivity was separated from free radioligand by rapid filtration through a 96-well GF/B Unifilter apparatus (PerkinElmer Life and Analytical Sciences, Courtaboeuf, France) and quantified using a TopCount scintillation counter (PerkinElmer).

Calcium mobilization: *h*GPR54-expressing CHO cells were loaded with 2.5 μM Fluo-4 AM in the presence of 2.5 mM probenecid, as described previously.[9a] Compounds were serially diluted in 10 mM HEPES pH 7.4, 0.4 mM NaH_2PO_4, 137.5 mM NaCl, 1.25 mM $MgCl_2$, 1.25 mM $CaCl_2$, 6 mM KCl, 10 mM glucose and 1 mg mL^{-1} BSA). Agonist-evoked increases in intracellular calcium were recorded over time (5 s intervals over 220 s) at 37 °C through fluorescence emission at 520 nm (excitation at 485 nm) by using a Flexstation III (Molecular Devices, Sunnyvale, CA, USA). Peak response amplitudes were normalized to basal and maximal (cells permeabilized with 20 μM digitonin) fluorescence levels.

In vitro metabolic stability in rat liver microsomes: All incubations were performed individually for each test compound. Compounds **3**, **26**, **27**, and **47** (50 μM) were incubated at 37 °C under standard incubation conditions: phosphate buffer (pH 7.4), NADPH (1 mM), and rat liver microsomes (0.1 mg protein). The final concentration of DMSO was 0.5%. At different times (0, 5, 15, 30, and 45 min), aliquots (35 μL) of the reaction mixture were quenched with 100 μL of cold acetonitrile. Upon centrifugation of the resulting mixture, supernatants were analyzed by a generic HPLC method performed on a Dionex Ultimate 3000 (Waters XSelect CSH C_{18} column, 5 μm, 4.6×50 mm) with the following parameters: Flow rate: 1 mL min^{-1}, column temperature: 30 °C, solvent system: A (MeOH) and B (0.05% TFA in H_2O), t=0–12 min, 20→100% A then t=12–15 min, 100% A. The ratio of product was determined by integration of UV spectra recorded at 202 nm. Metabolic stability was determined by the disappearance of the tested compound over time. The natural logarithm linear plots of the percent compound remaining based on chromatographic peak area versus time were plotted, and the slope was calculated by linear fitting of the curve. The microsomal intrinsic clearance [CL_{int}, expressed in μL min^{-1} (mg protein)$^{-1}$] was calculated using the equation:[17]

$$CL_{int} = \frac{0.693 \times (\text{incubation volume})}{t_{1/2} \times (\text{mg microsomal protein})} \quad (1)$$

In vitro metabolic stability in rat serum: All incubations were performed individually for each test compound. Compounds **3**, **26**, **27**, and **47** (50 μM) were incubated at 37 °C in rat serum (200 μL). Final concentration of DMSO was 0.5%. At different times (0, 15, 30, 60, and 120 min), aliquots (35 μL) of the reaction mixture were quenched with 100 μL of cold acetonitrile. Upon centrifugation of the resulting mixture, supernatants were analyzed by a generic HPLC method performed on a Dionex Ultimate 3000 (Waters XSelect CSH C_{18} column, 5 μm, 4.6×50 mm) with the following parameters: Flow rate: 1 mL min^{-1}, column temperature: 30 °C, solvent system: A (MeOH) and B (0.05% TFA in H_2O), t=0–12 min, 20→100% A then t=12–15 min, 100% A. The ratio of product was determined by integration of UV spectra recorded at 202 nm. Metabolic stability was determined by the disappearance of the tested compound over time. The natural logarithm linear plots of the percent of compound remaining based on chromatographic peak area versus time were plotted, and the slope was calculated by linear fitting of the curve. The in vitro half-life ($t_{1/2}$, min) was estimated by using 0.693/k, in which k is the biotransformation rate constant and corresponds to the slope of the ln-linear curve.

Effect of GPR54 agonists on circulating testosterone in adult male rats: In vivo assay conditions were essentially as described previously.[18] Briefly, the mice (n=6) received an intraperitoneal (i.p.) injection of the test compounds (kisspeptin, **3**, and **27** dissolved in ethanol/Ringer's solution [1:1]) at a dose of 10 mg kg^{-1} (where Ringer's solution consists of 6.5 mg mL^{-1} NaCl, 0.42 mg mL^{-1} KCl, 0.25 mg mL^{-1} $CaCl_2$, and 0.2 mg mL^{-1} $NaHCO_3$). Trunk blood was collected after decapitation 30 min after treatment. The blood was centrifuged for 10 min at 3000 rpm, and serum was collected and stored at −20 °C until hormone levels were determined. Free serum testosterone was measured using a direct radioimmunoassay (RIA) kit (DPC coat-a-count RIA method; Siemens Medical Solutions, Mölndal, Sweden).

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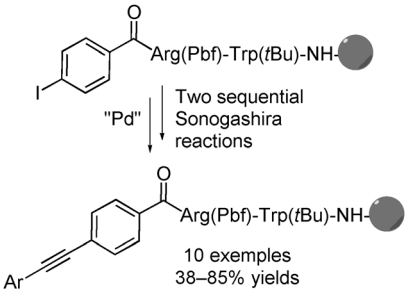
The simpler, the better: By using palladium-catalyzed reactions on supported peptides, we synthesized a series of dipeptides that act as agonists of the human KiSS1-derived peptide receptor (*h*GPR54). However, we showed that the simple N-benzoylated dipeptide Bz-RW-NH_2 is sufficient to induce a significant increase in levels of circulating testosterone when administered to rats at a dose of 10 mg kg^{-1} (i.p.).



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Development of Dipeptidic *h*GPR54 Agonists