

Side Chain Orientation of Tryptophan Analogues Determines Agonism and Inverse Agonism in Short Ghrelin Peptides

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We describe two synthetic amino acids with inverted side chain stereochemistry, which induce opposite biological activity. Phe⁴ is an important part of the activation motif of ghrelin, and in short peptide inverse agonists such as KwFwLL-NH₂, the aromatic core is necessary for inactivation of the receptor. To restrict indole/phenyl mobility and simultaneously strengthen the interaction between peptide and receptor, we exchanged the natural monoaryl amino acids for diaryl amino acids derived from tryptophan. By standard solid-phase peptide syn-

thesis, each of them was inserted into ghrelin or in the aromatic core of the inverse agonist. Both ghrelin analogues showed nanomolar activity, indicating sufficient space to accommodate the additional side chain. In contrast, diaryl amino acids in the inverse agonist had considerable influence on receptor signaling. Whereas the introduction of Wsf maintains inverse agonism of the peptide, Wrf shifts the receptor more to active states and can induce agonism depending on its introduction site.

Introduction

The ghrelin receptor is a G protein-coupled receptor with a basal activity of 50% of the signaling induced by its endogenous ligand ghrelin.^[1] Due to this ligand-independent activity, the receptor can be targeted by two different kind of ligands: agonists can further increase the activity, and inverse agonists decrease basal activity.^[2] As the receptor plays a major role in energy homeostasis, growth hormone release and alcohol abuse, it is an interesting therapeutic target.^[3] However, substantial knowledge of the interaction between the receptor and ligand is still missing, which would allow the design of specific ligands and to decrease the side effects of therapeutics.

The interaction between ghrelin and its receptor was investigated in detail in the last few years. Ghrelin(1–4) is necessary for receptor activation, although binding affinity is two orders of magnitude lower than that of full-length ghrelin.^[4] It is assumed that the Ser³ lipidation with octanoic acid is important

for membrane interaction.^[5] In a structural model of ghrelin bound to its receptor, it was proposed to extend this minimal binding motif up to His⁹.^[6] Therein, amino acids 1 to 5 and His⁹ and Arg¹¹ directly interact with the receptor. The N-terminal motif binds with the central cavity in the ghrelin receptor, and the α -helix with His⁹ interacts with ECL3. Phe⁴ is close to glutamine in transmembrane helix three (Gln120^{3,29}) and isoleucine in helix four (Ile178^{4,60}).

Constitutive activity of the ghrelin receptor is based on the presence of aromatic locks in the receptor. Phe279^{6,51}, Phe309^{7,39}, and Phe312^{7,42} form an aromatic cluster that was described to be crucial by Holst et al. in 2004.^[1a] Furthermore, Trp276^{6,48} of the CWxP motif plays an important role in constitutive activity and interacts with Phe221^{5,47} to stabilize active conformations.^[7] Moreover, His280^{6,52} is also thought to be relevant in constitutive activity by interacting with the two amino acids and Arg283^{6,55}.^[8] The hexapeptide KwFwLL-NH₂ binds with its N-terminus deep in the receptor binding pocket; D-Trp² is located close to the aromatic phenylalanine cluster and can disrupt this interaction.^[9] Substitution of D-tryptophan by the slightly bulkier 1-naphthyl and 3-benzothienyl functionalities resulted in increased activity and decreased constitutive activity. In contrast, agonism can be induced by exchanging D-tryptophan at position 4 with D-2-naphthylalanine.^[10]

KwFwLL-NH₂ is remarkably sensitive to small structural changes, and Phe⁴ of ghrelin is important for receptor interaction.^[6,10,11] Thus, we introduce a pair of diastereomeric β -phenyl tryptophans as shown in Figure 1 in these two peptides. Synthetic methods for the construction of enantiopure β , β -diaryl amino acids have advanced in the past couple of years and are becoming increasingly relevant in the modification of peptides.^[12] The side chains of phenylalanine and tryptophan were combined to obtain Wrf and Wsf. The analogues

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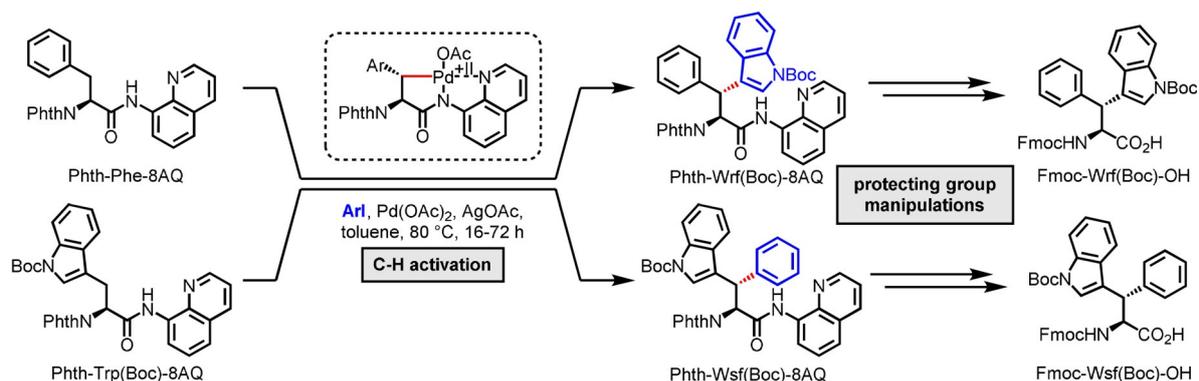


Figure 1. CH activation of Phe and Trp leads to the β,β -diarylated amino acids Wrf and Wsf. Adequately protected precursors are activated as *trans*-substituted palladium complexes. The addition of aryl iodides finalizes the cross-coupling (red bonds). Naming in the three-letter code is based on the amino acid with the higher side chain priority (W = L-tryptophan). The second letter indicates the side chain stereochemistry *R* or *S* of the stereoisomers, and the third letter stands for the one-letter code of the second amino acid.

were synthesized using CH activation and introduced in the peptide by solid-phase peptide synthesis. Interestingly, these space-demanding side chains affect activity when introduced at peptide positions important for receptor interaction only slightly in ghrelin analogues, but significant effects were found for the signaling of the small inverse agonist.

Results and Discussion

Synthesis and analysis of diaryl amino acids with naturally occurring side chains

Wrf and Wsf were obtained by C β arylation of adequately protected Phe and Trp precursors, respectively. For Wrf, L-Phe was N-terminally protected with a phthaloyl (Phth) group, C-terminally modified with 8-aminoquinoline (8AQ) and side chain protected with *tert*-butyloxycarbonyl (Boc). The crucial synthetic step was the C–C bond formation that requires a directing group like 8AQ to form the palladium complex necessary for diastereoselective C β arylation.^[13] The diastereoselective β -indoylation of racemic Phth-Phe-8AQ gave *rac*-Phth-Wrf/wsf-8AQ, which was crystallized and examined with X-ray analysis (Figure 2).

The asymmetric cell contains both enantiomers with an anti-periplanar arrangement of H α and H β that minimizes steric strain around the C α –C β bond. Consequently, the Phe moiety of Phth-Wrf(Boc)-8AQ (1) assumes a χ_1 angle of -41.4° , while

the Trp moiety is found to be -163.8° . The indolyl group is oriented with $\chi_2 = 39.7^\circ$.

In solution, the indolyl group of Phth-Wrf(Boc)-8AQ assumes an analogous orientation as in the crystal as determined from NOEs and 3J coupling constants as shown in Figure 3. In spite of the congested environment, the phenyl ring shows the AMM'XX' spin system expected for a mobile aromatic ring with a low barrier of rotation about the C β –C γ bond of the phenyl ring. H2 of the indole moiety is oriented toward H α in Phth-Wrf(Boc)-8AQ and Phth-Wsf(Boc)-8AQ (2), resulting in a “mirrored” presentation of important motive for receptor interaction.

The phenylation of Phth-Trp(Boc)-8AQ yielded Phth-Wsf(Boc)-8AQ. The χ_1 rotation of H α and H β is anti-periplanar like the diastereomer Phth-Wrf(Boc)-8AQ. The orientation of the Phe moiety is anti-periplanar with respect to the highest priority substituent (NPhth), whereas the Trp moiety is oriented gauche minus with a χ_2 angle of about 120° of the indole ring. In conclusion, the conformational analysis shows that the indolyl moiety assumes a “mirrored” orientation in Wsf relative to Wrf, which results in an opposite presentation of the important indole motif toward the receptor. The conformation of both building blocks is best visualized by the Newman projections of the amino acids. The χ_2 rotation is fixed for the Trp moiety while Phe shows high rotational mobility for both amino acids. The effect for Phe is similar to the so-called “steric gearing”, which is well studied for two $6-\pi$ -aromatic rings linked to the same carbon wherein ground state destabiliza-

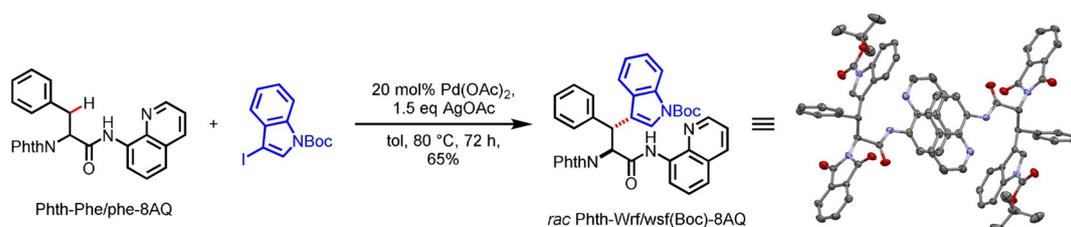


Figure 2. The indolyl group is introduced by CH activation in a palladium complex. The crystal structure (CCDC 1903900) of racemic Phth-Wrf/wsf(Boc)-8AQ shows a pair of enantiomers in the asymmetric unit with a Wrf shows the V-shaped alignment of the two aryl side chains. The Newman plot shows the orientation about the C α –C β bond.

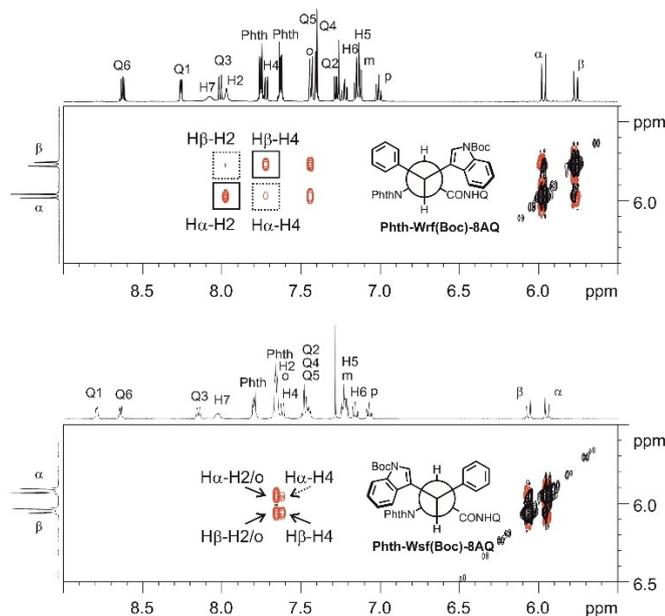


Figure 3. NMR spectra of Phth-Wrf(Boc)-8AQ (500 MHz, 300 K, CHCl_3); bottom: ROESY spectrum of Phth-Wsf(Boc)-8AQ (500 MHz, 300 K, CHCl_3). The expansion from the ROESY spectra of both building blocks Phth-Wrf(Boc)-8AQ and Phth-Wsf(Boc)-8AQ identify the preferred conformation in solution. In both cases, the large 3J coupling constant of approximately 12 Hz between $\text{H}\alpha$ and $\text{H}\beta$ indicate an antiperiplanar orientation. Strong NOE contacts are indicated with boxes and dashes, respectively, and weak NOE contacts are depicted by dotted boxes and dashes. o, m, p are the phenyl ring spin system which shows high rotational mobility. Q stands for the 8AQ directing group and H for the indole ring.

tion caused by the close proximity of the two rings lowers the rotational barrier in spite of the steric crowding.^[14]

The CH arylation of enantiopure amino acids followed by protecting group manipulations^[13] yielded Fmoc-Wrf(Boc)-OH (**3**) and Fmoc-Wsf(Boc)-OH (**4**), which were then used in solid-phase peptide synthesis for the assembly of the ghrelin analogues.

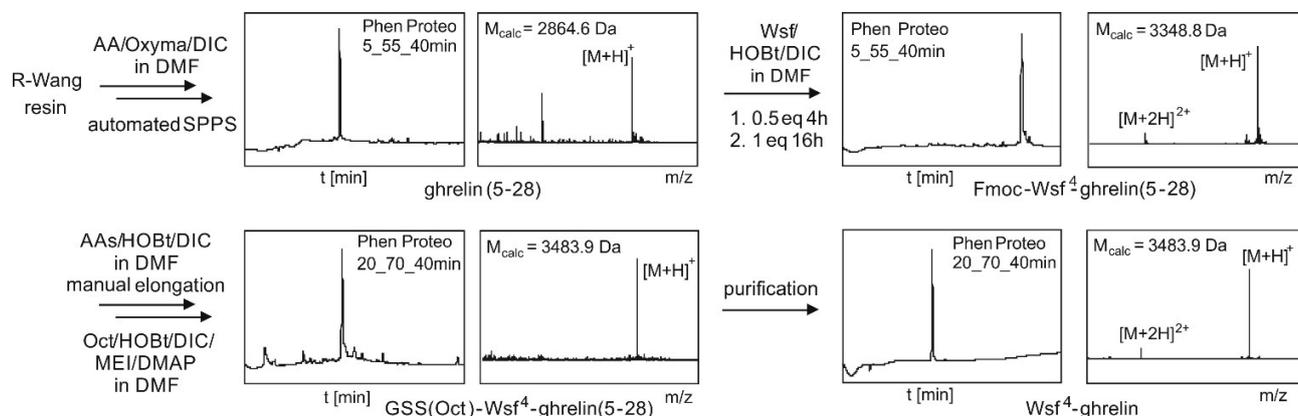


Figure 4. Fmoc/*t*Bu solid-phase peptide synthesis of Wsf^4 -ghrelin (**7**). Synthesis was carried out on a Arg(Pbf)-Wang resin. Ghrelin(5–28) was synthesized by automated SPPS in a MultiSynTech Syrol. Fmoc-Wsf(Boc)-OH was introduced manually with DIC and HOBt with 0.5 equiv for 4 h and with 1 equiv for 16 h. Next, the peptide was elongated with Fmoc-Ser(Trt)-OH, Fmoc-Ser(*t*Bu)-OH, and Boc-Gly-OH. Trt was removed and octanoic acid coupled with DIC/HOBt/MEI/DMAP. Following purification, a peptide with purity > 95% was obtained and the identity was confirmed by mass spectrometry.

Incorporation into peptides by solid-phase peptide synthesis

The diaryl amino acids were introduced in the peptide backbone. We chose ghrelin (**5**) and the ghrelin receptor inverse agonist KwFwLL-NH₂ (**8**) as both peptides contain Phe and Trp that are important for receptor activity. Phe⁴ in ghrelin and either *D*-Trp², Phe³ or *D*-Trp⁴ in the inverse agonist were substituted by Wrf or Wsf. The diaryl amino acids were coupled by standard solid-phase peptide synthesis. Exemplarily, this is shown with analytics for Wsf^4 -ghrelin (**7**) in Figure 4.

We have successfully incorporated the diaryl amino acids in the sequence. After automated synthesis of ghrelin(5–28), Wsf was coupled with DIC and HOBt. To decrease the amount of building block, reaction was carried out twice with 0.5 equiv for 4 h and 1 equiv overnight, respectively. MS and RP-HPLC analysis of intermediates showed high coupling efficiency, and a shift in hydrophobicity could be observed in RP-HPLC following Wsf coupling. The same has been found for the Wrf analogue. After N-terminal elongation and coupling of octanoic acid, the peptide was purified. Full cleavage of the peptide demonstrated high coupling efficiencies for all amino acids. Following purification, all peptides were analyzed on two different RP-HPLC columns and by MALDI-ToF mass spectrometry. Purity of > 95% and correct identity was observed for all compounds (Table 1).

In the past, β,β -modified amino acids were included in the peptide backbone, as Dip (β,β -diphenyl alanine) at position 2 in the short lead peptide.^[10] However to our knowledge, no peptides with stereoisomeric β,β -diaryl amino acids containing natural side chains were introduced into a peptide before. Comparing the retention times of the lead peptides with diaryl amino acids analogues, it is notable that there are no clear changes in the retention time. It can be assumed that the additional phenyl or indole group does not influence the hydrophobicity of the peptides in a great extent.

Table 1. Peptide analytics. All compounds were examined for their identity by MALDI-ToF mass spectrometry and purity on two different columns.

No.	Peptide ^[a]	M_{theo} [Da]	M_{exp} [M+H] ⁺	R_t [%B] ^[b]	R_t [%B] ^[c]	Purity [%]
5	ghrelin ^[10]	3368.9	3369.9	40.3	33.9 ^[e]	> 95
6	Wrf ⁴ -ghrelin	3483.9	3845.0	40.6	33.0	> 95
7	Wsf ⁴ -ghrelin	3483.9	3845.0	40.4	32.8	> 95
8	KwFwLL-NH ₂ ^[10]	890.5	891.6	56.6	45.4 ^[d]	> 95
9	K-Wrf-FwLL-NH ₂	966.6	967.6	55.0	44.6	> 95
10	K-Wsf-FwLL-NH ₂	966.6	967.6	54.7	44.3	> 95
11	Kw-Wrf-wLL-NH ₂	1005.6	1006.5	58.0	47.5	> 95
12	Kw-Wsf-wLL-NH ₂	1005.6	1006.5	58.7	47.9	> 95
13	KwF-Wrf-LL-NH ₂	966.6	967.6	55.3	44.3	> 95
14	KwF-Wsf-LL-NH ₂	966.6	967.5	54.4	43.1	> 95
15	KbFwLL-NH ₂ ^[10]	907.5	908.5	58.8	47.8 ^[d]	> 95
16	Kb-Wrf-wLL-NH ₂	1022.5	1023.4	61.0	50.7	> 95
17	Kb-Wsf-wLL-NH ₂	1022.5	1023.4	61.0	51.0	> 95
18	AwFn-Isn-NH ₂ ^[15]	729.4	730.4	53.3	54.2 ^[e]	> 95
19	KwFnLL-NH ₂ ^[10]	901.5	902.5	58.5	48.4	> 95
20	AwF-Wrf-Isn-NH ₂	794.4	795.5	52.0	45.0	> 95
21	AwF-Wsf-Isn-NH ₂	794.4	795.4	53.2	43.5	> 95

[a] b = D-3-benzothienyl alanine, n = D-2-naphthyl alanine, Isn = isonipecotic acid. [b] Jupiter Proteo (Phenomenex: 250 × 4.6 mm; 4 μm; 90 Å). [c] Kinetex Biphenyl 100 Å (Phenomenex: 250 × 4.6 mm; 5 μm; 100 Å), if not indicated otherwise. [d] Aeris Peptide 100 Å (Phenomenex: 250 × 4.6 mm; 3.6 μm; 100 Å). [e] VariTide RPC (Varian: 250 × 4.6 mm; 6 μm; 200 Å).

Introduction of diaryl amino acids had considerable influence on peptide behavior

All analogues were tested in an IP-One assay to evaluate the activity of the analogues relative to the lead. COS7 cells stably transfected with the ghrelin receptor C-terminally tagged with eYFP were used and incubated with different ligand concentrations for 3 h. Ghrelin (5) had a potency of 2.8 nM, which is in the range of previous studies.^[10,16] KwFwLL-NH₂ (8) and KbFwLL-NH₂ (b = 3-benzothienyl alanine, 15) were used as control and EC₅₀ values of 183 nM and 22.9 nM were observed, respectively. Compared with radioactive IP accumulation assays, both peptides possessed an approximately 10-fold reduced activity in the Cisbio IP-One assay.^[10] However, other groups that used the assay could obtain similar EC₅₀ values as found in this study for KwFwLL-NH₂.^[16]

Wrf⁴- and Wsf⁴-ghrelin (6 and 7) behaved similar in the cell studies with a potency loss of about 12-fold (Figure 5A). However, efficacy was similar to ghrelin (Table 2). As position 4 is

part of the minimal binding motif of ghrelin to its receptor,^[6] it can be concluded that the binding pocket is not so narrow around Phe⁴ and there is space for an additional aromatic group as the peptide is still able to activate the receptor. Also the orientation seems to be not important as both analogues 6 and 7 show comparable activity.

Van Craenenbroeck et al. investigated Phe⁴ by Ala and Tyr substitution in ghrelin(1–14) analogues.^[17] While Ala⁴ showed no activity in an IP accumulation assay and significantly reduced activity in Ca²⁺ assay compared to ghrelin(1–14), this loss of activity and affinity could be restored partially by Tyr⁴. This underlines the importance of an aromatic group at this position. As Phe⁴ is close to Gln120^{3,29} and Ile178^{4,60},^[6] interactions may be based primarily on hydrophobic interactions.

First generation of ghrelin inverse agonist peptides containing diaryl amino acids were based on KwFwLL-NH₂ (8) and Wrf or Wsf were introduced into the aromatic core. To evaluate inverse agonism and agonism, the behavior and E_{max} (decreased or enhanced receptor efficacy) of the peptide is of interest.

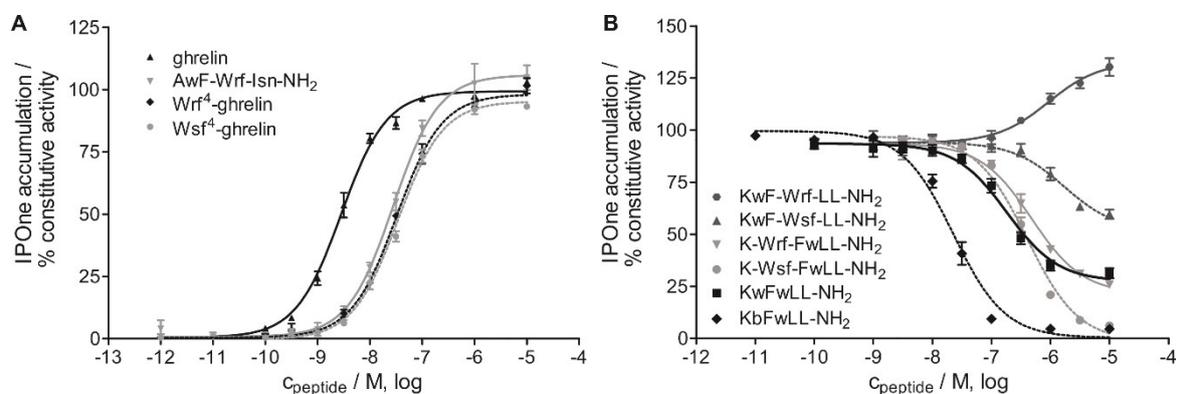


Figure 5. IP-One assays of Wrf/Wsf analogues. The assay was carried out in COS7 cells stably transfected with the ghrelin receptor. Data are the mean ± SEM of ≥ 2 independent experiments performed in triplicates. A) Ghrelin analogues and the short Wrf peptide show about 15-fold lower activity than the endogenous ligand. B) Orientation of the diaryl amino acid determines agonism or inverse agonism for short peptides.

Table 2. Activity data from IP-One assay.^[a]

No.	EC ₅₀ [nM]	pEC ₅₀	E _{max} [Δ%]	Behavior
5	2.8	8.55 ± 0.04	99 ± 2	agonist
6	32.6	7.49 ± 0.03	98 ± 2	agonist
7	34.2	7.47 ± 0.03	96 ± 2	agonist
8	183	6.74 ± 0.07	66 ± 3	inverse agonist
9	480	6.32 ± 0.06	72 ± 2	inverse agonist
10	405	6.39 ± 0.05	99 ± 3	inverse agonist
11	149	6.83 ± 0.13	55 ± 4	inverse agonist
12	283	6.55 ± 0.09	77 ± 4	inverse agonist
13	919	6.04 ± 0.13	39 ± 3	agonist
14	> 1000	–	–	inverse agonist
15	22.9	7.64 ± 0.04	99 ± 2	inverse agonist
16	549	6.26 ± 0.10	56 ± 3	inverse agonist
17	> 1000	–	–	inverse agonist
18	16.4	7.78 ± 0.10	110 ± 6	agonist
19	228	6.64 ± 0.05	93 ± 2	agonist
20	27.9	7.56 ± 0.06	105 ± 3	agonist
21	82.8	7.08 ± 0.05	95 ± 2	agonist

[a] pEC₅₀ and E_{max} values are the mean ± SEM of at least two independent experiments; E_{max} is normalized to ghrelin (5) efficacy for ghrelin analogues and multiple modified agonists and normalized to KbFwLL-NH₂ (15) for inverse agonists and 13.

Substitution of D-Trp² with Wrf (9) and Wsf (10) resulted in inverse agonists with a slight decrease in activity relative to 8. However, the efficacy was increased. Especially Wsf² (10) showed full inverse agonist efficacy, that is, the peptide is able to fully shift the receptor to the inactive state. Notably, Wsf is still 18-fold less active than 15 with 3-benzothienyl side chain at position 2. Wrf³ (11) activates the receptor similar to the lead peptide with decreased inverse agonist efficacy, and Wsf³ (12) showed an increased inverse agonist efficacy. The substitution of D-Trp⁴ had a more severe influence than the other two positions. Whereas Wsf (14) introduction resulted in a more than 10-fold decrease in inverse agonist activity, Wrf (13) completely diminished the inverse agonist effect. The peptide behavior was switched to agonism with low but detectable activity (Figure 5B). It is known from previous studies that position 4 can be used to induce agonism characteristics into the peptide.^[10] However, here the shift between agonism and inverse agonism is based just on the configuration of the side chains of Wrf and Wsf. In general, Wsf-containing peptides shift the receptor more to inactive states and Wrf to active states. Wsf peptides have higher inverse agonist efficacies for all monosubstituted analogues relative to the corresponding Wrf analogue. Interestingly, SAR studies of the inverse agonist [D-Arg¹,D-Phe⁵,D-Trp^{7,9},Leu¹¹]-substance P, on which the short lead peptide is based, demonstrated that the substitution of D-Trp⁹ (same as D-Trp⁴ in KwFwLL-NH₂) with Ala and L-Trp completely diminished the inverse agonist activity.^[18] This is in contrast to the findings in this study. However, the additional phenyl side chain may regain the lost activity. Leu⁵ of KbFwLL-NH₂ was recently replaced by amino acids with various characteristics and it was shown that Lys, Gln, Phe, and Ala can induce agonism.^[11a] The most efficient agonist was the D-2Nal⁵ analogue, whereas 2Nal⁵ had no activity at the ghrelin receptor. In contrast, the introduction of either L-Trp or D-Trp at this

position did not influence the peptide behavior, the peptide remained an inverse agonist. This supports the idea that direction and orientation of the aromatic side chains in short ghrelin peptides decide between agonism and inverse agonism.

To further investigate the influence of the diaryl amino acids, a second generation of peptides was synthesized. The full inverse agonist KbFwLL-NH₂ (15) was modified at position 3 with Wrf (16) or Wsf (17). Whereas the combination of D-Bth and Wrf was similar to Kw-Wrf-wLL-NH₂ (11), introduction of Wsf significantly decreased the activity of the inverse agonist. Interestingly, similar to the single modification of KwFwLL-NH₂, there seems to be a tendency that the Wsf-containing peptide is a stronger inverse agonist than the Wrf compound. In addition, Wrf⁴/Wsf⁴ were studied in more detail. For this, AwFn-Isn-NH₂ (n = D-2-naphthyl alanine, Isn = isonipecotic acid, 18) and KwFnLL-NH₂ (19) were used as comparison. We reported earlier that the short ghrelin ligands bind the receptor with its N-terminus facing into the binding pocket of the ghrelin receptor and it was proposed for KwFnLL-NH₂ (19) that Phe³ and D-2Nal⁴ can interact with each other.^[9,10] This interaction might be stronger for Wrf due to the restrictive relative orientation of its two aryl side chains. Therefore, the introduction into a known potent agonist was of interest. In IP-One assay, EC₅₀ values of 16.4 nM and 228 nM were observed for 18 and 19, respectively. The EC₅₀ value of 18 is similar to previous studies.^[15] However, a 10-fold shift has been observed for 19, similar to the inverse agonist. In addition, we observed super-agonism for 19 in a radioactive IP accumulation assay but not in the Cisbio IPOne assays. When Wrf was included into AwFn-Isn-NH₂ substituting D-2Nal (20), potency was almost the same as that of 18. The corresponding Wsf analogue 21 had a slightly lower potency and efficacy than 20.

Interestingly, the D-2Nal⁴ analogue 19 was 4-fold more active than the Wrf⁴ analogue (13), but the differences were diminished in the combined agonist. D-2Nal possesses strong agonism-prone characteristics at position 4 and can even turn the efficient inverse agonist KbFwLL-NH₂ (15) into an agonist by substitution of Leu⁵.^[11a] It is surprising that 20 can almost reach the same activity as 18. Additionally, the use of the stereoisomeric β,β-diaryl amino acids demonstrates that an additional aromatic group is not disturbing this interaction.

Conclusions

Understanding the binding and activation mechanism of G protein-coupled receptors is one of the most important tasks to facilitate drug development. We introduced stereoisomeric β,β-diaryl amino acids in the binding motif of ghrelin and the short ghrelin inverse agonist KwFwLL-NH₂ to evaluate the activation profile. Diaryl amino acids are a powerful tool as they combine two side chains in different orientation and can help discuss interaction patterns. Here, we demonstrated that Phe⁴-ghrelin can be modified with Trp/Phe analogues and still possesses nanomolar activity and full efficacy. Additionally, the sensitivity of the aromatic core of a short ghrelin receptor inverse agonist was confirmed and our studies display that the orientation of the side chains in the stereoisomeric β,β-diaryl

amino acids decides on the agonism tendencies of the peptides. Wrf shifts peptide behavior toward agonism, whereas Wsf with inverted side chain stereochemistry is prone to inverse agonism.

Experimental Section

Synthesis of β,β -diaryl amino acids

Phth-Wrf(Boc)-8AQ. A pressure flask was charged with 6.00 g (14.2 mmol, 1.0 equiv) Phth-Phe-8AQ (97% ee), 24.4 g (71.2 mmol, 5.0 equiv) *N*-Boc-3-iodoindole, 639 mg (2.85 mmol, 20 mol%) Pd(OAc)₂ and 3.56 g (21.4 mmol, 1.5 equiv) AgOAc. The flask was flushed with Ar. To this was added 12.0 mL of anhydrous toluene and the mixture was vigorously stirred at 80 °C for 72 h. After completion, the mixture was diluted with CH₂Cl₂ and filtered through a pad of Celite. The residue was concentrated and purified by column chromatography on silica using toluene/ethyl acetate to furnish 5.86 g (9.20 mmol, 65%) of Phth-Wrf(Boc)-8AQ (d.r. > 25:1, 97% ee) as a white solid among with 19.4 g (56.5 mmol, 79%) of re-isolated aryl halide as a pale brown oil.

Phth-Wsf(Boc)-8AQ. A pressure flask was charged with 5.00 g (8.92 mmol, 1.0 equiv) Phth-Trp(Boc)-8AQ, 4.0 mL (35.7 mmol, 4.0 equiv) iodobenzene, 200 mg (0.89 mmol, 10 mol%) Pd(OAc)₂ and 2.23 g (13.4 mmol, 1.5 equiv) AgOAc. The flask was flushed with Ar. To this was added 5.0 mL of anhydrous toluene and the mixture was vigorously stirred at 80 °C for 16 h. After completion, the mixture was diluted with CH₂Cl₂ and filtered through a pad of Celite. The residue was concentrated and purified by column chromatography on silica using toluene/ethyl acetate to furnish 4.04 g (6.35 mmol, 71%) of Phth-Wsf(Boc)-8AQ (d.r. > 25:1) as a white solid.

Analysis of building blocks can be found in the Supporting Information. Protecting group manipulations were carried out as described previously.^[13]

Peptide synthesis

Fmoc/tBu solid-phase peptide synthesis of ghrelin and the hexapeptides was performed on solid support with an automated multiple peptide synthesizer (Syrol, MultiSynTech, Bochum, Germany) and standard Fmoc-protected amino acids (Orpegen OPC, Heidelberg, Germany).^[11a] Manual synthesis was performed with 5 equiv Fmoc amino acid (Iris Biotech, Marktredwitz, Germany), 5 equiv DIC (Iris Biotech) and 5 equiv HOBt (Iris Biotech) in DMF. Ghrelin analogues were synthesized on Fmoc-Arg(Pbf)-Wang resin (Iris Biotech). Ser³ side chain was protected with Trt and Gly¹ N-terminally Boc-protected to ensure specific modification with octanoic acid following Trt cleavage with TFA/TIS/CH₂Cl₂ (1:5:94, v/v/v) by using 5 equiv octanoic acid, 5 equiv DIC and 5 equiv HOBt, 5 equiv methylimidazole and 0.1 equiv DMAP in DMF. Hexapeptide analogues were synthesized at Rink amide resin (Iris Biotech). β,β -diaryl amino acids Fmoc-Wrf(Boc)-OH and Fmoc-Wsf(Boc)-OH were introduced in the peptide sequence with first 0.5 equiv Fmoc-protected amino acid, 0.5 equiv DIC and 0.5 equiv HOBt in DMF for 4 h and second 1 equiv Fmoc-protected amino acid, 1 equiv DIC and 1 equiv HOBt in DMF for 16 h. Cleavage from the resin and of the side chains was carried out with TFA/TA/EDT (90:7:3, v/v/v) and deprotected peptide was precipitated with ice-cold mixture of hexane/diethyl ether (3:1, v/v). Purification of the peptides was achieved with preparative HPLC on a reversed-phase C18 column (Phenomenex AeriS Peptide 5u XB-C18: 250×21.2 mm; 5 μ m; 100 Å) with a flow

rate of 15 mL min⁻¹ and λ =220 nm. A linear gradient of solvent B in solvent A (solvent A: water + 0.1% TFA, solvent B: acetonitrile + 0.08% TFA) was used depending on the peptides. Purity of the peptides was determined by analytical reversed-phase HPLC on at least two of the following columns: Jupiter Proteo (Phenomenex: 250×4.6 mm; 4 μ m; 90 Å), Kinetex Biphenyl 100 Å (Phenomenex: 250×4.6 mm; 5 μ m; 100 Å), AeriS Peptide 100 Å (Phenomenex: 250×4.6 mm; 3.6 μ m; 100 Å) or VariTide RPC (Varian: 250×4.6 mm; 6 μ m; 200 Å). A linear gradient of 20–70% B in A in 40 min (solvent A: water+0.1% TFA, solvent B: acetonitrile+0.08% TFA) at 40 °C with a flow rate of 0.6 mL min⁻¹ to 1.55 mL min⁻¹ was used depending on the column (λ =220 nm). Peptides were analyzed by MALDI-MS (UltraflexIII, Bruker, Bremen, Germany). The observed masses were in full agreement with the calculated masses and peptides with a purity \geq 95% could be obtained according to the analytical HPLC.

Cell culture and IP-One assay

COS7 cells were stably transfected with the ghrelin receptor fused C-terminally to eYFP and cultured in a humidified atmosphere at 37 °C and 5% CO₂ in Dulbecco's modified Eagle's medium with higher glucose (Lonza, Basel, Switzerland) supplemented with 10% (v/v) FCS (Biochrom, Berlin, Germany) and 0.4 mg mL⁻¹ hygromycin B (Invivogen, Toulouse, France). For evaluation of the activity, Cisbio IP-One Gq assay kit was used according to previous description with alterations.^[19] A standard curve was prepared in HBSS with 10 mM LiCl to determine the linear range of the assay and to convert obtained HTRF ratio to IP1 concentrations. 10 000 cells per well were seeded out in a 384-well flat white plate and cultured overnight. Stimulation was carried out in triplicates for 3 h. Peptides were solved in DMSO and diluted with HBSS containing 10 mM LiCl (maximal DMSO content 1%). Subsequently, 3 μ L IP1-d2 and 3 μ L Ab-cryptate in lysis buffer were added and incubated on a tumbler for 60 min. Fluorescence was measured at 620 nm and 665 nm. HTRF ratio was calculated as the ratio 665/620. Obtained data were analyzed with GraphPad Prism 5.0 (GraphPad Software, San Diego, USA). Hexapeptide analogues with a single modification as well as inverse agonists were normalized to KbfwLL-NH₂. Ghrelin analogues and multiple substituted agonists were normalized to ghrelin for better comparison. E_{\max} is the efficacy of the peptide and represents the difference between constitutive activity and activity at maximal effect of the peptide. EC_{50} is the peptide concentration at half-maximal effect.

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Conflict of interest

The authors declare no conflict of interest.

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