# Oligoproline helices as structurally defined scaffolds for oligomeric G protein-coupled receptor ligands†

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***Received 10th November 2009, Accepted 21st January 2010***
***First published as an Advance Article on the web 22nd February 2010***
**DOI: 10.1039/b923556f**



Oligoprolines (OPs) are used as rigid backbone scaffolds for the design of oligomeric ligands that target specific G protein-coupled receptors. The OPs were designed to vary in length, the position and number of the ligand-functionalized residues incorporated. For all synthesized compounds a typical PP type II helix was evidenced by circular dichroism indicating that decoration of the helix with large ligands did not affect the helical conformation. Pharmacological evaluation revealed that oligomerization of an agonist with the use of an oligoproline scaffold showed an increase in potency when compared to the monomeric counterparts.

## Introduction

Proline oligomers composed of three or more proline residues adopt well-defined helical structures,[1] and are integral parts of many proteins found in nature where they partake in protein folding and protein–protein interactions.[2] Artificial oligoproline helices have found application as molecular rulers, for instance to correlate the distance of a fluorophore and a quencher molecule with the efficiency of resonance energy transfer.[3] Related research describes the design of amphiphilic molecules based on oligoproline helices by the incorporation of modified (hydrophilic or hydrophobic) proline residues at specific sites of the oligomers.[4] Oligoprolines may exist in two helical forms that are easily distinguished by circular dichroism (CD) measurements. PP type II helices (PPII) are most common and are formed when the amide bonds connecting the proline residues have the *trans* configuration.[1] PPII helices have three proline residues per turn with a rise of 3.1 Å per residue. Oligoprolines assembled from modified proline residues having an electron-withdrawing substituent at C4 (fluorine,[5] azide[6]) appear to equilibrate towards *cis* amide bonds and the resulting PP type I (PPI) helices have 3.3 proline residues per turn with a rise of 1.9 Å per residue.

The secondary structure provided by oligoproline helices, which are maintained in aqueous solutions in which they normally dissolve well, indicates that oligoproline helices may be useful scaffolds onto which two or more copies of a receptor ligand can be grafted. The resulting dimeric and oligomeric structures may find application in the study of ligand–receptor interactions in which multivalency is thought to be of importance for potency and/or selectivity.

In this respect, data has amassed recently that point towards dimerization and oligomerization of G-protein coupled receptors (GPCRs) as physiologically relevant in signal transduction.[7] Oligomerization of weak or moderately selective GPCR ligands therefore appears an attractive strategy to obtain compounds with improved pharmacological activity.[8] A case in point and the focus of the here presented studies is the thienopyrimydyl derivative, **Org 43553** (Scheme 1),[9] a potent agonist of the human luteinizing hormone receptor (LHR) that also exerts considerable agonistic activity on the human follicle-stimulating hormone receptor (FSHR).[9c] Both these GPCRs are glycoprotein hormone receptors that play a fundamental role in the human reproductive system. LHR activation induces male testosterone production and ovulation in females,[10] whereas FSHR activation stimulates female ovarian follicle growth and spermatogenesis in males.[11]

## Synthesis and structural evaluation

We established that thienopyrimidine **LHA** exerts agonistic activity on the LHR and FSHR with a similar potency compared to lead compound **Org 43553** (Table 1).[12] **LHA** contains an acetylenic moiety that can be used in a reaction with a set of oligoprolines (OPs) containing several 4-azidoproline (Azp) residues. The azidoproline-containing helices were prepared by standard Fmoc solid-phase peptide-synthesis by incorporation of one or more copies of either (4*R*)-4-azidoproline or (4*S*)-4-azidoproline[13] at various positions in the sequence. Proline helices encompassing one or more copies of the pharmacophore were readily assembled by a copper(I)-catalysed Huisgen [2+3] cycloaddition of **LHA** with the azidoproline oligomers. A representative example of the synthesis of compound **9*S*-Azp** and **9*S*-LHA$_2$** is depicted in Scheme 1. In this fashion, we prepared a panel of 26 (oligo)proline derivatives of which the final compositions are depicted in Table 1.

The helical distribution of the compounds can be evidenced by circular dichroism (CD) experiments. PPI type helices are

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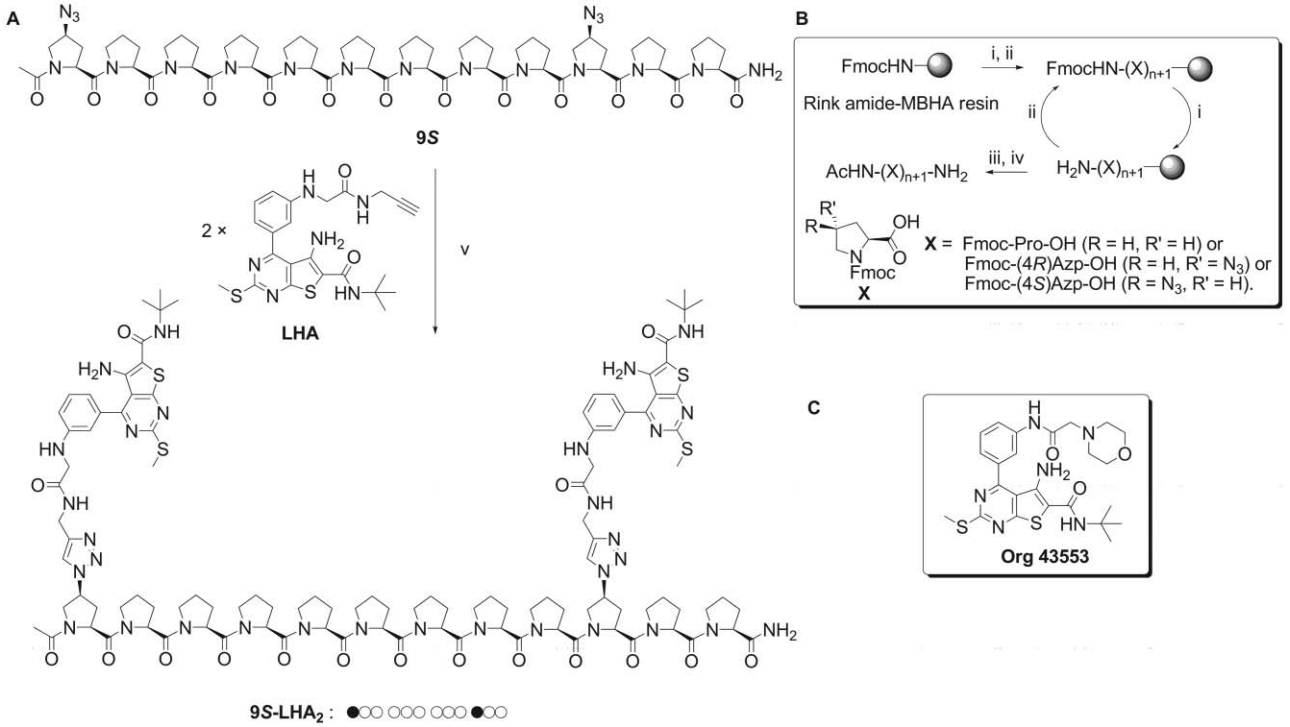
† Electronic supplementary information (ESI) available: Experimental procedures and additional characterization. See DOI: 10.1039/b923556f




**Scheme 1** A. Representative example for the preparation of ligands containing LHR agonist (**LHA**) by the copper catalyzed [2+3]-Huisgen cycloaddition. ○ represents proline; ● represents LHA functionalized proline. B. Synthetic scheme of the oligoproline scaffolds. *Reagents and conditions*: *i*. 20% piperidine/DMF, 0.5 h; *ii*. Fmoc-Pro-OH or Fmoc-Azp-OH, HCTU, DiPEA, NMP, 3 h; *iii*. $Ac_2O$, DiPEA, DMF, 2 h; *iv*. 95% TFA/$H_2O$, 1 h; *v*. Sodium ascorbate (5 eq), $CuSO_4$ (1 eq), *t*BuOH/$CH_3CN$–$H_2O$. C. Structure of lead compound **Org 43553**.

characterized by a minimum at 230 nm and a maximum at 212 nm in their CD spectra, while a PPII type helix has its maximum at 225 nm and a minimum at 207 nm. CD spectra were recorded for all compounds, both the ligand-functionalized derivatives from Table 1 and their azidoproline containing precursors. In all cases a spectrum indicative for PP type II helix was observed, independent of the spacer length and substitution pattern when measured in a 10% *i*PrOH/phosphate buffer (see for a representative CD spectrum that of **9*S*-LHA$_2$**, Table 2).[14] We also found that the relative numbers populating the PPII conformation as opposed to other (PPI) conformations differed from one compound to the next. It is generally observed that compounds with more stable PP type II helices have more intense ellipticities at 207 nm than compounds that equilibrate faster with PP type I configuration.[15] Compounds **5–9** all possess polyproline helices of similar lengths. Within these series, a more intense ellipticity of the (4*R*)-azidoproline containing helices was observed, compared to the corresponding (4*S*)-Azp containing helices (Figure 1). This is in agreement with the results described by Wennemers *et al.*[6] The reverse trend is observed when the helices are substituted with a LHA ligand. Here, the (4*R*)-LHAs have less intense ellipticities than the (4*S*)-LHAs. This may indicate that the triazole substituted proline may destabilize the PPII conformation for the compounds containing (*R*)-configured 4-substituted proline derivatives.

Another trend that can be seen in Table 2 is that the constructs in where the substituents are grouped close together have more intense ellipticities than those in which they are further apart (compare compounds **5** to **9**). This holds especially true for the helices containing the LHA pharmacophores. It may be of interest to investigate whether this is only due to the size of the substituent or that other factors play a role.

## Biological evaluation

Biological evaluation revealed that all compounds are potent LHR agonists whereas their potency to agonize the FSHR appeared at most rather modest (Table 1). Some general trends are observed. For example, compounds bearing two copies of the pharmacophore are two to five times more potent LHR agonists compared to the compounds **1–4** that are equipped with a single copy of the parent compound. The compounds incorporating more than two pharmacophores (**15*R*-LHA$_3$** and **15*S*-LHA$_3$**) are LHR agonists in the low nM range. Of importance is the observation that all compounds are weaker LHR and FSHR agonists compared to the parent compounds **Org 43553** and **LHA**. This trend is evident also in the oligoproline series **1–4** indicating the penalty of attaching an oligoproline moiety onto the pharmacophore. Looking at the oligomeric series **5–14** there is no clear selectivity trend discernible but we do note the high LHR selectivity of **15*S*-LHA$_4$** (FSHR/LHR = 188), a result that may be attributed to the incorporation of multiple pharmacophores. Also apparent, and pointing towards an explanation for the enhancement in selectivity, is that whereas FSHR agonistic activity for all compounds is at least a tenfold lower compared to the lead compound (**Org43533**), agonistic activities towards LHR vary much more. For instance, **15*S*-LHA$_4$** agonizes the LHR with an $EC_{50}$ of 9 nM, which is only two- to threefold less potent



**Table 1** Mean agonistic potency ($EC_{50}$) and selectivity for the LHR and FSHR. All compounds are full agonists on the LHR and partial agonists on the FSHR. The mean $EC_{50}$ are calculated from the –log $EC_{50}$ values from two or three independent experiments performed in duplicate. The SD of $pEC_{50}$ is generally lower than 0.2. ○ represents proline; ● represents LHA functionalized proline

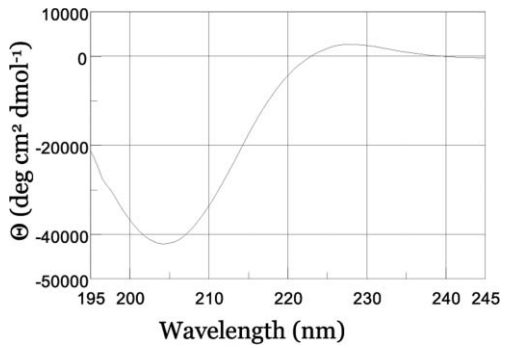
| | $EC_{50}$/nM | | | | | $EC_{50}$/nM | | |
|---|---|---|---|---|---|---|---|---|
| | FSHR | LHR | FSHR/LHR[a] | *Compound* | | FSHR | LHR | FSHR/LHR[a] |
| | | | | ***Monomeric*** | | | | |
| **Org 43553**[b] | 110 | 3.7 | 28 | | | | | |
| **LHA**[c] | 126 | 0.9 | 137 | | | | | |
| **1*R*-LHA** | 2109 | 32 | 67 | ●○○ ○○○ ○○○ ○○○ | **1*S*-LHA** | >3000 | 76 | 40 |
| **2*R*-LHA** | >3000 | 98 | >31 | ○○○ ●○○ ○○○ ○○○ | **2*S*-LHA** | >3000 | 110 | >27 |
| **3*R*-LHA** | >3000 | 107 | >28 | ○○○ ○○○ ●○○ ○○○ | **3*S*-LHA** | >3000 | 114 | >26 |
| **4*R*-LHA** | >3000 | 44 | >69 | ○○○ ○○○ ○○○ ●○○ | **4*S*-LHA** | >3000 | 84 | >36 |
| | | | | ***Dimeric*** | | | | |
| **5*R*-LHA$_2$** | nd | nd | nd | ●●○ ○○○ ○○○ ○○○ | **5*S*-LHA$_2$** | 2092 | 26 | 80 |
| **6*R*-LHA$_2$** | 1427 | 20 | 71 | ●○● ○○○ ○○○ ○○○ | **6*S*-LHA$_2$** | 2362 | 32 | 75 |
| **7*R*-LHA$_2$** | 1499 | 27 | 56 | ●○○ ●○○ ○○○ ○○○ | **7*S*-LHA$_2$** | 2367 | 30 | 80 |
| **8*R*-LHA$_2$** | 1498 | 29 | 51 | ●○○ ○○○ ●○○ ○○○ | **8*S*-LHA$_2$** | 1898 | 27 | 71 |
| **9*R*-LHA$_2$** | 1335 | 25 | 54 | ●○○ ○○○ ○○○ ●○○ | **9*S*-LHA$_2$** | 1178 | 20 | 60 |
| **10*R*-LHA$_2$** | 1223 | 16 | 76 | ●○○ ○○○ ○○○ ○○○ ●○○ | **10*S*-LHA$_2$** | nd | nd | nd |
| **11*R*-LHA$_2$** | 2193 | 27 | 82 | ●○○ ○○○ ○○○ ○○○ ○○○ ●○○ | **11*S*-LHA$_2$** | nd | nd | nd |
| **12*R*-LHA$_2$** | 1518 | 18 | 84 | ●○○ ○○○ ○○○ ○○○ ○○○ ○○○ ●○○ | **12*S*-LHA$_2$** | nd | nd | nd |
| **13*R*-LHA$_2$** | 2267 | 33 | 68 | ○○○ ●○○ ●○○ ○○○ | **13*S*-LHA$_2$** | 2707 | 32 | 84 |
| **14*R*-LHA$_2$** | 1999 | 31 | 65 | ○○○ ○○○ ●○○ ●○○ | **14*S*-LHA$_2$** | 1200 | 33 | 36 |
| | | | | ***Oligomeric*** | | | | |
| **15*R*-LHA$_4$** | 1067 | 9 | 119 | ●○○ ●○○ ●○○ ●○○ | **15*S*-LHA$_4$** | 1764 | 9 | 188 |

[a] Selectivity observed for the LHR ($EC_{50}$ FSHR/$EC_{50}$ LHR). [b] Taken from reference 9*c*. [c] Taken from reference 12. nd = not determined

compared with **Org43533**. It may thus be tentatively concluded that LHR agonism by ligand substituted oligoprolines depends on the nature of the oligomers (number of ligands, distance between these and possibly also the configuration of the 4-substituted prolines employed) whereas FSHR agonism is compromised in any case. Obviously, a more in-depth study employing more oligomers is needed to reach a definite conclusion in this. An intriguing question in this respect is also whether the oligomers bind to the receptors as PP type II helices and thus whether geometric restrictions in the oligomers make that two ligands can bind to the LHR (or a dimer) at the same time but cannot do so, or to a lesser extent, to the FSHR. One possibility to probe this would be to introduce non-proline residues at predesigned sites in the oligomers and establish the agonistic potency of these structures, with an enhanced intrinsic conformational flexibility, towards the LHR and FSHR.[16] The results presented here clearly demonstrate that oligoprolines equipped with two or more pharmacophores are useful starting points in the design of bioactive compounds with an improved selectivity profile.

In summary, we prepared a set of (4*R*)- or (4*S*)-4-azidoproline helices that were functionalized with a luteinizing hormone receptor agonist by means of a copper (1)-catalyzed Huisgen [2+3] cycloaddition. The resulting compounds adopted a PPII helical conformation as judged by circular dichroism, indicating that decoration of the helix with large agonists did not affect the helical conformation. Pharmacological evaluation revealed that functionalization of the lead compound with an oligoproline scaffold resulted in a drop in potency for the LHR. However, compared to the monomeric counterparts, a significant increase in potency on the LHR was observed that is related to the increase in LHA functionalized prolines on the helix. This result indicates the potential of oligoproline helices substituted at multiple sites with pharmacophores to obtain GPCR ligands to study receptor dimerization in more detail. Although we did not succeed in increasing the potency of the lead compound towards the LHR we obtained a compound that combines a strong potency (only two-threefold less compared to the lead compound) with a markedly enhanced selectivity. Given that next to potency selectivity of a compound towards the desired target is a highly important parameter in medicinal chemistry we feel that our approach, in which oligoprolines are used as scaffolds to bring together a number of pharmacophores holds promise for the development

**Table 2** The intensity in ellipticity observed for dimeric azidoproline containing compounds **5–9-Azp** and LHA functionalized proline compounds **5–9-LHA** at 205 nm is given in the Table. All compounds were measured at $4E^{-5}$ M in 10% *i*PrOH/phosphate buffer pH 7.2. A representative CD-spectra observed for the oligoproline compounds is depicted below. Here, compound **9*S*-LHA$_2$** shows a minimum at 205 nm and maximum at 228 nm




| | | Observed ellipticity at 205 nm ($\times 10^3$ deg cm$^2$ dmol$^{-1}$) | | | | |
|---|---|---|---|---|---|---|
| | | (*R*) | (*S*) | | (*R*) | (*S*) |
| ●●○ ○○○ ○○○ ○○○ | **5-Azp** | −30.2 | −28.5 | **5-LHA** | nd | −60.2 |
| ●○● ○○○ ○○○ ○○○ | **6-Azp** | −28.2 | −23.7 | **6-LHA** | −33.2 | −62.8 |
| ●○○ ●○○ ○○○ ○○○ | **7-Azp** | −28.1 | −27.7 | **7-LHA** | −29.6 | −49.1 |
| ●○○ ○○○ ●○○ ○○○ | **8-Azp** | −32.7 | −24.0 | **8-LHA** | −16.7 | −42.0 |
| ●○○ ○○○ ○○○ ●○○ | **9-Azp** | −24.3 | −11.9 | **9-LHA** | −10.0 | −42.1 |

of structurally and conformationally defined bi- and multivalent ligands.

## Acknowledgements

We are indebted to Dr A.J.W. Hsueh, Stanford University, for providing human LHR and FSHR cDNA. We thank Monique van Amstel and Robert Tan for expert technical assistance. This work was supported by the Netherlands Organization for Scientific Research (NWO).

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16 We thank the referee for this relevant and interesting suggestion.



 