RESEARCH ARTICLE

Revealing the Indispensable Role of the RFamide Functionality using a Novel Acid Labile Benzofuranone based Amine (ALBA) Linker

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The RFamide family of peptides represents an important class of GPCR ligand neuropeptides covering a wide range of biological functions. While many analogues of the highly conserved C-terminal RFamide motif within this peptide class have been synthesized and their functional significance elucidated, additional exploration of the structure activity relationship is of value. We have developed a novel linker for solid phase peptide synthesis (SPPS) which is able to anchor amine functionalised compounds for further elaboration. The acid labile benzofuranone based amine (ALBA) linker (5-(3-aminopropylcarbamoyl)-2-[[tert-butyl(diphenyl)silyl]oxymethyl]benzoic acid) is compatible with Fmoc based SPPS and has two cleavage modes. As a proof of concept, the ALBA linker was used to successfully synthesise a novel analogue of Kisspeptin 10, the natural ligand for GPCR54, whereby the natural RFamide motif was replaced with an RFamine. Biological evaluation of the amine-containing analogue revealed that the group is not compatible with receptor activation.

Keywords: cleavable linker, Kisspeptin, GPR54, GPCR, RFamide, solid phase peptide synthesis (SPPS).

Introduction

The RFamide family of neuropeptides are endogenous ligands of G-Protein Coupled Receptors (GPCRs), all of which possess a highly conserved C-terminal motif of arginine followed by amidated phenylalanine, from which they get their name. A host of RFamide peptides across 5 broad classes[1] have been identified in mammals and have been found to be involved in a vast array of biological processes with cross talk between them.[2–6] It is believed that there are more peptides, functions, and receptors yet to be discovered.[7] In a screen of 161 GPCR receptors in C. elegans against a panel of 344 peptides, 39 receptors were identified as being activated only by RFamide peptides,[8] demonstrating the value of these exciting biological entities. Due to the range of disease states these peptides are implicated in, information on how their structure affects binding to receptors is valuable in understanding binding modes and for the design and generation of active analogues. The importance of the C-terminal amide functionality in RFamide peptides such as Kisspeptin,[9,10] 26RFa[11] and neuropeptide FF[12] has been established by recent investigations, with Kisspeptin being arguably the most studied.[1] The results of these investigations suggest that changes to the amide deplete binding and subsequent activation of the receptor (See *Figure 1*). Modifications studied include; exchanging the amide for the corresponding carboxylic acid or hydrazide, methylation or dimethylation of the nitrogen, or replacement of the amide with a hydroxyl group. In
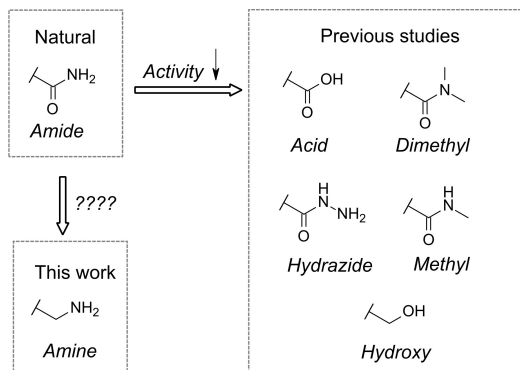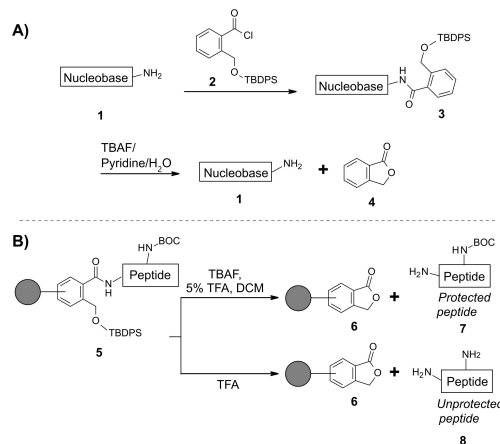
Figure 1. Reported modifications to the C-terminal amide of RFamide peptides, all of which lead to significantly decreased activity. In this work we assess the effect of replacing the amide with a primary amine.


Scheme 1. (A) Reported use of 2-HMBA in nucleobase protection (B) Proposed use of 2-HMBA as a linker. Two possible cleavage modes could enable generation of either the protected or unprotected peptide.

each case, activity is significantly diminished. We were interested in expanding this investigation by exploring an analogue of an RFamide peptide wherein the C-terminal amide is replaced with a primary amine. A compound of this type could have increased hydrophilicity, solubility, and *in vivo* stability.

Results and Discussion

In order to investigate the effects of an RFamide to RFamine transformation, we used Kisspeptin 10 (KP10), the endogenous ligand of GPR54, as a model system. KP10 is a decamer RFamide peptide of sequence YNWNSFGLRF-NH$_2$.[13] The simplest route to access this compound is by solid phase peptide synthesis (SPPS). This requires a cleavable linker system with the ability to anchor amine containing compounds, demonstrate stability to Fmoc based SPPS conditions and release the peptide following completion of synthesis. Cleavable linkers developed specifically for capture and release of amines have previously been reported,[14–17] however parallel work within our group which utilised the cyclisation of (hydroxymethyl)benzoic acid to form benzofuranone inspired us to investigate this system as a cleavable linker, potentially expanding the available linker toolbox. In depth reviews of general linker chemistry and their characteristics are beyond the scope of this article but may be found in literature.[18]

The benzofuranone system has been described in previous work by Dreef-Tromp et al.[19] and Kuijpers et al.[20] for the protection of amine groups in nucleosides (*Scheme 1A*), Here, the hydroxymethyl group of 2-HMBA was protected with tert-butyldiphenylsilyl

chloride (TBDPS—Cl), preventing premature cyclisation under neutral conditions (compound **2**), before conjugating to the nucleobase amine (**3**). During deprotection, fluoride is employed for TBDPS removal, then basic conditions encourage intramolecular cyclisation, forming benzofuranone **4**, and regenerating the deprotected nucleobase **1**. It was hypothesised that if a handle for attachment to a solid support were introduced, this system could be used for anchoring and elaboration of amine containing compounds (**5**). Two different cleavage modes could then be exploited; neat TFA to simultaneously remove the TBDPS group, any acid sensitive side chain protecting groups (e.g. Boc, tBu) and promote cleavage of the compound from solid support (**8**), or a milder method where fluoride is used to remove TBDMS and dilute TFA is used for linker cleavage, potentially allowing release of compounds with acid sensitive protecting groups in tact (**7**).

ALBA Linker Synthesis

Synthesis of the novel linker was accomplished in three steps (*Scheme 2*). 5-carboxyphthalide **9** was coupled with 3-azidopropylamine to give compound **10**. Subsequent opening of the phthalide ring with hydroxide, then *in situ* protection of the resulting alcohol **11** using tert-butyldiphenyl silyl chloride[21] furnished completed acid labile benzofuranone based amine (ALBA) linker **12 (5-(3-aminopropylcarbamoyl)-2-[[tert-butyl(diphenyl)silyl]oxymethyl]benzoic acid)**. As attachment of the linker to solid support *via*
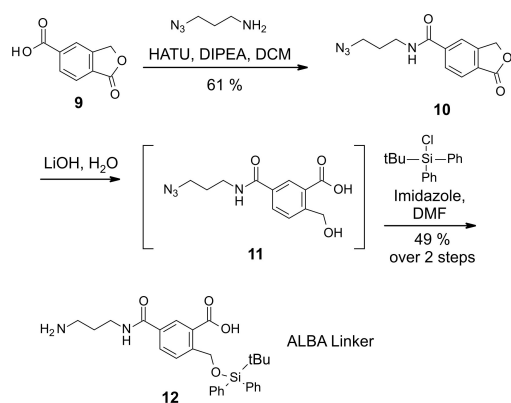
Scheme 2. Synthesis of the ALBA linker.

amide coupling is precluded due to the presence of a free carboxylic acid in the final compound, incorporation of an azide handle was chosen, as this allows attachment of the linker to resin *via* Huisgen dipolar cycloaddition. The resulting triazole linkage may be preferable to some other commonly used functionalities such as ethers, which can produce unwanted side reactions during acidic cleavage.[22]

Linker Cleavage Tests

The ALBA linker was loaded with tryptamine in solution and assessed in proof-of-concept cleavage studies (*Table S1*). The linker proved stable to Fmoc based SPPS coupling conditions (*Table S1*, entry 1) and Fmoc deprotection conditions (*Table S1*, entry 2). Cleavage of the linker to liberate tryptamine was next investigated. First, TFA was employed, as this is known to remove TBDMS groups and so has the potential to deprotect the hydroxymethyl group and simultaneously encourage cyclisation to form the benzofuranone moiety. This process was shown to be successful and went to completion within 4 hours in neat TFA (*Table S1*, entry 4). A milder approach to compound cleavage was also investigated, by first deprotecting the hydroxymethyl group using fluoride then subsequently encouraging cyclisation in less harsh conditions. The TBDPS group was fully deprotected after 30 minutes using TBAF, however subsequent cyclisation and elimination of tryptamine in basic conditions (pyridine/H2O) was not observed after 24 hours (*Table S1*, entry 5). 2-HMBA can be cyclised using mild acidic conditions[14] (5 % TFA in DCM), therefore this was assessed in our system and these conditions yielded the fully released tryptamine within 4 hours (*Table S1*, entry 6). Having the option to utilise these

milder cleavage conditions could also allow liberation of peptides with side chain protecting groups intact.
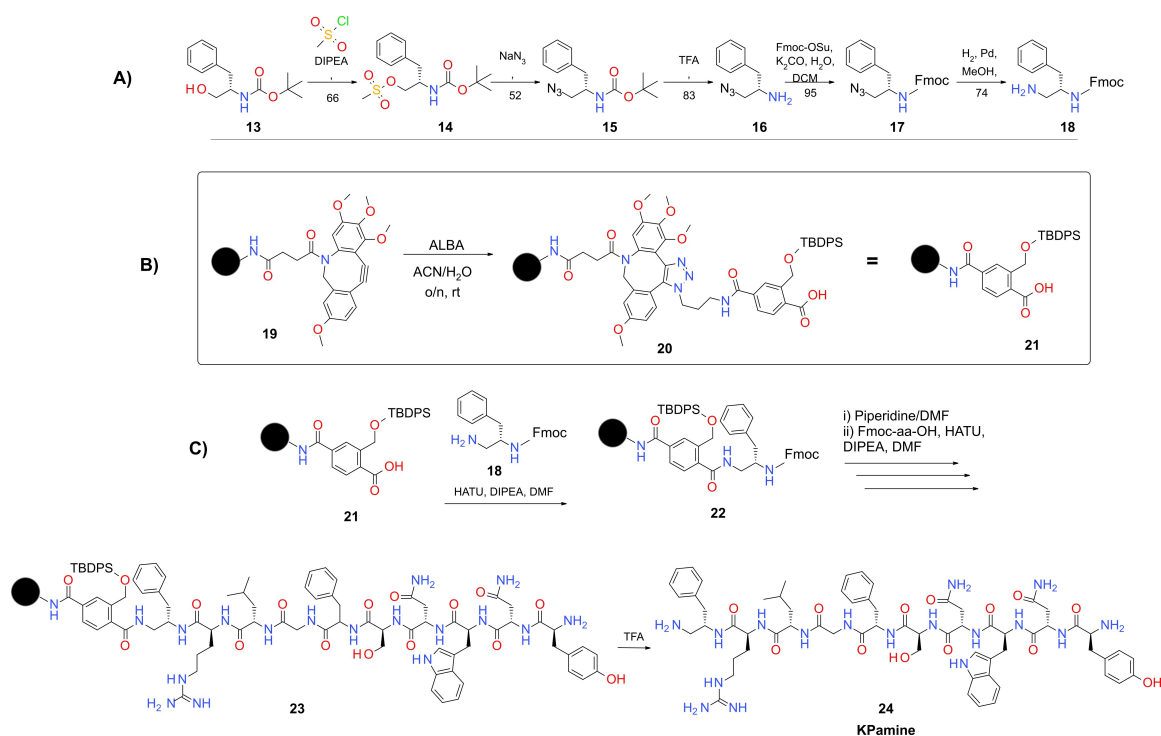
Synthesis of the KP10amine

Encouraged by these results, we returned our attention to producing the KP10amine analogue. The required C-terminal amine was introduced by synthesis of a phenylalanine analogue whereby the carboxylic acid is replaced with a primary amine (*Scheme 3A*). Commercially available Boc-l-phenylalinol was employed to generate mesylate **14**. Sodium azide displacement of the mesyl group generated azide **15** in good yields. Exchanging the *N*-Boc protecting group for Fmoc ensured compatibility with SPPS. Finally, reduction of the azide (in acidic conditions to preserve the Fmoc group) gave the desired amine functionalised analogues of phenylalanine (**18**).

With the ALBA linker and amine building block in hand, synthesis of the Kisspeptin 10 analogue was undertaken. Tentagel NH2 beads were loaded with a strained cyclooctyne[23] (*Scheme 3B*) to give the immobilised alkyne **19**, allowing the ALBA linker to be loaded using copper free Huisgen 1,3 dipolar "click" cycloaddition, generating resin **20**. An advantage of this method is that no other reagents are required for coupling, so any unused linker material can be recovered simply by evaporation of the solvent. This allows a large excess of the linker to be used to ensure high reaction yields. The amine building block **18** was then coupled to the linker and Fmoc SPPS was used the build-up of the full KP10amine decamer **23**. Following synthesis, release of the final product from the solid support was achieved by incubating the resin in a mixture of TFA and scavengers for four hours and the peptide was purified by RP-HPLC.

Evaluation of KP10amine

The KP10amine (**24**) was functionally tested on COS-7 cells stably transfected with GPR54. As the receptor signals through a Gq pathway, the total inositol triphosphate produced in cells following incubation with compounds was measured, which correlates to receptor signalling (see *Supporting Information* for details). *Figure 2* shows that the KP10amine derivative does not induce receptor signalling at submicromolar concentrations (*Figure 2*), indicating that compound **24** has severely reduced activity on GPR54 triggering compared to wild type KP10. This result reiterates the importance of conservation of the RFamide motif in Kisspeptin 10.

Scheme 3. A) Synthesis of amine containing building block. B) A strained cyclooctyne allows loading of the ALBA linker to solid support *via* copper free click. C) SPPS of the Kisspeptin 10 analogue on the ALBA linker. Acidic cleavage furnishes the desired *C*-terminal amine peptide.
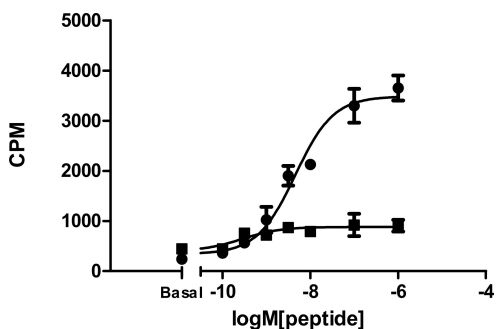

Figure 2. EC50 activation curves for wild type Kisspeptin 10 (circles) and the KP10amine analogue (squares). The KP10amine analogue does not activate GPR54, suggesting a pivotal role for the amide group in this RFamide peptide.

In order to determine a tentative rationale for this reduction in activity, a model of GPR54 was obtained from the AlphaFold service, into which the Kisspeptin 10 peptide was docked. See *Supporting Information* for methods. Given the predicted nature of the protein model and that some conformation change is likely to result upon peptide binding, a good fit was observed between the peptide and the protein pocket in the final docked pose. The C-terminus was placed at the terminus of the pocket, where it formed a hydro-phobic cul-de-sac lined by Val126, Tyr207, Leu212, Phe272, Trp276, Ile-279 and Tyr313. Only two polar residues interrupt this hydrophobic surface: Gln122 and Gln280 (*Figure 3*). Both were observed to be within range of the peptide C-terminus to form hydrogen bonds: Gln122 with the amine group, and Gln280 with the carbonyl group. Although this structural model is highly tentative, relying as it does on computational prediction for both the protein and the peptide ligand, it is consistent with the activity data and suggests high importance of both of these functional groups.

Beyond the RFamide family of peptides, searching the ChEMBL[24] database (version 33) revealed only one directly comparable *C*-terminal amide to amine modification. The CHEMBL5084213 molecule containing a C-terminal amine moiety is noted to bind the Escherichia coli sliding clamp with a dissociation constant K_D of 877 ± 45 nM.[25] The C-terminal amide derivative is noted to improve this K_D to 268 ± 68 nM. An amine functionalized C-terminus is also present in other peptidic molecules without directly comparable amide derivatives, see *Supporting Information* accompanying this manuscript for further information. Most interestingly, the modification is noted in nature, with the antibacterial peptides cicadapeptin I, and cicada-
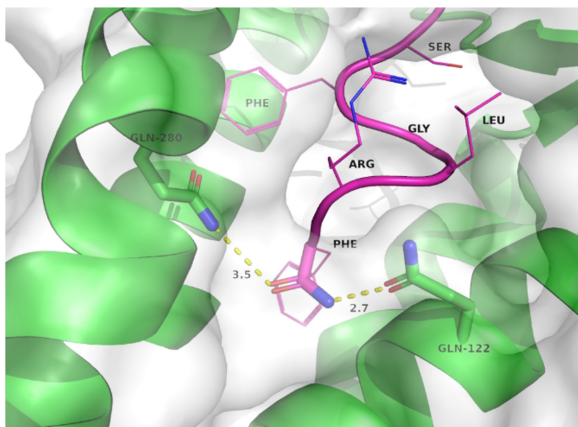
Figure 3. Model of GPR54 with the kisspeptin 10 peptide bound. GPR54 is shown as green secondary structure, and a cutaway translucent white surface. The side chains of Gln122 and Gln280 are represented by stick models. The Kisspeptin 10 peptide is shown in magenta, with the backbone displayed as a ribbon, and side chains as lines. Polar functional groups of the C-terminus are shown as stick models. Nitrogen atoms are colored blue, and oxygens red. Hydrogens are omitted for clarity. Putative hydrogen bonds between these groups and the two Gln residues are shown as dashed yellow lines of length 3.5 and 2.7 Å.

peptin II found in the insect killing and parasitic fungus Cordycepsheteropoda.[26] See *Supporting Information* accompanying this manuscript for a description of cheminformatics techniques, code and substructure searches run to extract molecules.

Conclusions

In conclusion, a novel ALBA linker which enables anchoring of amines onto solid support was developed which is compatible with Fmoc solid phase peptide synthesis. This linker has two cleavage modes, allowing the release of unprotected peptides and a milder cleavage method, which could allow compound cleavage while leaving selected acid sensitive protecting groups intact. The ALBA linker was used to generate a C-terminal amine analogue of Kisspeptin 10, which was tested in a functional assay against GPR54. The inability of this compound to activate the receptor and the structural rationale for activity ablation further demonstrates the importance of conservation of the amide moiety in the C-terminal RFamide motif. Further applications of the ALBA linker are currently being investigated.

Experimental Section

Experimental details are provided in the *Supporting Information.*

Supporting Information

The authors have cited additional references within the *Supporting Information.*[24–38] *Supporting Information* accompanying this manuscript describes biological assays, chemical synthesis (building blocks, peptide, linker and copper-free click reagent, SPS methods), cleavage tests, and QC data.

Author Contribution Statement

GM and MA conceived the project, GM performed chemical synthesis, MH and RPM performed biological experiments, SS and DH performed cheminformatics analysis and peptide modelling, MA provided funding. All authors contributed to manuscript preparation and proofing.

Acknowledgements

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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