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# MODIFICATIONS OF THE 4, 4'-RESIDUES AND SAR STUDIES OF BIPHALIN, A HIGHLY POTENT OPIOID RECEPTOR ACTIVE PEPTIDE

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**Abstract**: Modifications of 4,4' residues of Biphalin have resulted in greater binding selectivity and biological potency for the μ opioid receptor. A higher partition coefficient across the phospholipid bilayer membrane has been achieved by using a β-branched unusual amino acids.


One of the central goals of modern peptide and protein chemistry is to develop better approaches for understanding the relationships between peptide structure, conformation, topography and dynamics, and various biological functions by designing peptide molecules with specific topographical and conformational features. Such conformational structure-biological activity relationship studies have been of critical importance for studies of peptide molecular design and molecular recognition processes in biological systems.[1-9]

A major achievement in the area of opioid research during the past two decades has been the demonstration of multiple opioid receptors including δ, μ, and κ opioid receptors.[10,11] The identification of endogenous peptide ligands for these receptors such as the enkephalins[12] has led to developments in the design of selective, biologically stable peptide molecules with enhanced potency. Most of the opioid peptide ligands prepared to date are structurally derived from enkephalins.[13,14]

The first generation of the dimeric peptides were derived from enkephalins and had structures of the type Tyr-D-Ala-Gly-Phe-NH in which n = 0-12.[15-21] One of the original efforts for designing these peptides

Tyr-D-Ala-Gly-Phe-HN-$(CH_2)_n$

was to probe the hyposeses that different receptors might be clustered in a small portion of the plasma membrane and that such molecules would serve as a probe for such receptors. Biphalin (n = 0, (Tyr-D-Ala-Gly-Phe-NH)$_2$) is a highly potent peptide for both δ and μ opioid receptors. More importantly, research in our laboratories has shown that Biphalin is 257- and 6.7-fold more potent than morphine and etorphine, respectively, in eliciting antinociception.[22] Its unusual antinociceptive profile suggests a potentially novel mechanism of a action that involves some kind of synergestic interaction between μ and $\delta_2$ opioid receptor types or subtypes. Biphalin may thus represent the first in a series of such compounds, which may lead to significant new therapeutic advantages.[22]

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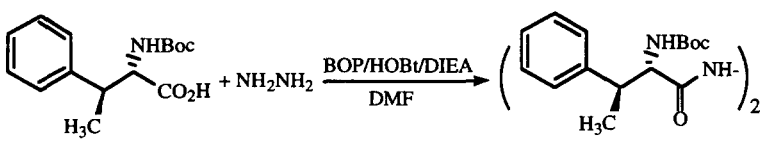
In order to determine the structural reasons for its unusual biological profile, we have sought to develop analogues with greater selectivities for μ and δ receptors. In this report, we wish to disclose our results of modifying the 4 and 4' positions with topographically constrained amino acids that provide new insights into the conformational structure-biological activities properties of Biphalin.

**Peptide Synthesis and Purification**

The β-branched aromatic α-amino acids used in this study were stereoselectively synthesized by new methods developed in our laboratory using an Evans-type auxiliary as a chiral resolution reagent,[23] or by a 1, 2-asymmetric *cis* induction reaction.[24] Other unusual $N^{\alpha}$-Boc amino acids were purchased from Synthetech, Inc. (Albany, OR). Biphalin derivatives were obtained by solution phase synthesis using a convergent synthetic strategy. The wing precursor (Boc-Tyr-D-Ala-Gly-OH) was synthesized by a modified method reported previously.[16] The bridge dimers were synthesized by a new one-pot cross coupling reaction of hydrazine (1 equiv) and $N^{\alpha}$-Boc amino acids (2 equiv). BOP reagent(benzotriazol-1-yloxy-tris(dimethyl-amino)phosphonium hexafluorophosphate) or HBTU reagent ([2- (1H-benzotriazol-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophophate) was used for the cross coupling reaction in a solution of diisopropylethylamine and dimethylformamide. High yields (66%-98%) were obtained. The cross coupling reaction is demonstrated below for the synthesis of one of β-methylphenylalanine hydrazide dimers (Scheme 1).




**Scheme 1.** Cross coupling reaction for bridge dimer synthesis

Other cross coupling reactions were conducted between bridged dimers (1 equiv) and the tripeptide precursors (2 equiv) to yield the corresponding $N^{\alpha}$-Boc protected Biphalin derivatives. The resulting products were then deprotected by HCl-HOAc solution (4 M). The synthesized analogues were purified by RP-HPLC (linear gradient of 10-90% acetonitrile in 0.1% TFA in water over 40 min) and characterized by FAB-MS and amino acid analysis. The purity of the products were assessed by HPLC (one single peak, UV detection at 280 and 225 nm using two different linear gradients).

**Results and Discussions**

Opioid receptor binding affinity and selectivity ratios in competition binding assays with [$^3$H]CTOP(μ) and [$^3$H][p-ClPhe$^4$]DPDPE(δ) using guinea pig brain homogenates are listed in Table 1. Binding data using brain tissue often can provide a more accurate picture of the peptide ligand selectivity than bioassay data, especially for examing effects on brain mediated antinociceptive activity. The selectivities of these in this study are discussed below.

**Table 1.** Binding Affinities and Selectivities of Biphalin Analogues

| Biphalin Analogues | Binding Affinity $IC_{50}$ | | Selectivity μ/δ |
|---|---|---|---|
| | δ[a] | μ[b] | |
| **1** (Tyr-D-Ala-Gly-Phe-NH)$_2$ | 5.2 ± 0.3[c] | 2.8 ± 0.4[c] | 0.54 |
| **2** (Tyr-D-Ala-Gly-(2S,3R)β-Me-Phe-NH)$_2$ | 110 ± 13 | 1.3 ± 0.19 | 0.012 |
| **3** (Tyr-D-Ala-Gly-(2S,3S)β-Me-Phe-NH)$_2$ | 11 ± 1.7 | 3.0 ± 1.0 | 0.27 |
| **4** (Tyr-D-Ala-Gly-1'-Nal-NH)$_2$ | 6.4 ± 2.6 | 0.79 ± 0.16 | 0.12 |
| **5** (Tyr-D-Ala-Gly-2'-Nal-NH)$_2$ | 7.4 ± 1.9 | 1.7 ± 0.52 | 0.23 |
| **6** (Tyr-D-Ala-Gly-$F_5$Phe-NH)$_2$ | 7.8 ± 2.5 | 0.91 ± 0.21 | 0.12 |

a: versus [$^3$H][p-Cl-Phe$^4$]DPDPE; b: versus [$^3$H]CTOP; c: estimated from $K_i$

The results in Table 1 show that all of these topographical modifications of the Phe$^4$ and Phe$^{4'}$ residues have resulted in higher selectivities for the μ opioid receptor, and in addition, the binding affinities also have been improved (or remained unchanged). The (2*S*,3*R*)-β-methylphenylalanine analogue **2** was 45 times more selective ($IC_{50}$ = 1.3 nM for μ and $IC_{50}$ = 110 nM for δ) than native Biphalin, and is among the most μ-receptor selective Biphalin derivatives examined thus far. On the other hand, (2*S*,3*S*) β-methylphenylalanine modification **3**, resulted in only a two-fold enhanced selectivity relative to biphalin ($IC_{50}$ = 3.0 nM for μ and $IC_{50}$ = 11 nM for δ) analogue than over the native Biphalin, while the binding affinity to the μ opioid receptor remained unchanged. These results suggest that Biphalin selectivity can be enhanced by topographical constraints in the side-chain moieties, and that the (2*S*,3*R*) *threo*-L stereochemistry in the 4 and 4' phenylalanine positions is favorable for specific stereochemical interactions with the active sites on μ-opioid receptor.

Since the 4,4' aromatic positions have been shown to be important for Biphalin bioactivity, we, therefore, incorporated three other unusual amino acids(1-naphthylalanine, 2-naphthylalanine and pentafluorophenylalanine) into Biphalin which can change the conformation and topography of the Biphalin structure. The results in Table 1 show that the 1-Nal and 2-Nal 4,4'-substituted analogues, and the analogue with an aromatic moiety with low electron density (the $F_5$Phe$^4$-containing analogue **6**) can result in greater binding selectivities and increased binding affinities for μ opioid receptors relative to biphalin. Interestingly, Biphalin analogues which prefer the δ receptors were obtained when *para*-substituted phenylalanine-4 analogues with electron-withdrawing groups (*para* $NO_2$ and F) were used for 4,4'-modifications.[25] It seems that both *para* electron-withdrawing and *para* electron-donating groups are not desirable for design of μ receptor selective Biphalins.[25]

**Table 2.** Bioassay Results of Biphalin Analogues

| Biphalin Analogues | Bioassay data $IC_{50}$(nM)±SEM | | Ratio of GPI/MVD |
|---|---|---|---|
| | GPI(μ) | MVD(δ) | |
| **1** (Tyr-D-Ala-Gly-Phe-NH)$_2$ | 8.8 ± 0.3 | 27 ± 1.5 | 0.33 |
| **2** (Tyr-D-Ala-Gly-(2S,3R)β-Me-Phe-NH)$_2$ | 21 ± 7.7 | 180 ± 78 | 0.12 |
| **3** (Tyr-D-Ala-Gly-(2S,3S)β-Me-Phe-NH)$_2$ | 41 ± 17 | 120 ± 73 | 0.34 |
| **4** (Tyr-D-Ala-Gly-1'-Nal-NH)$_2$ | 1.7 ± 0.33 | 17 ± 3.5 | 0.10 |
| **5** (Tyr-D-Ala-Gly-2'-Nal-NH)$_2$ | 2.2 ± 0.56 | 9.3 ± 2.4 | 0.24 |
| **6** (Tyr-D-Ala-Gly-$F_5$Phe-NH)$_2$ | 8.9 ± 2.0 | 25 ± 4.1 | 0.36 |

The in vitro biological activities from the guinea pig ileum (GPI, for μ receptor) and mouse vas deferens (MVD, for δ receptor) assays are given in Table 2. Both the (2*S*,3*R*)- and (2*S*,3*S*)-β-methylphenylalanine-**4** substituted analogues **2** and **3**, though they bind as well or better than biphalin, had lower potency at the μ receptor (21.1 and 40.9 nM respectively) than biphalin, and a much lower potency in the δ assay (MVD 182 and 116 nM, respectively) as compared to native Biphalin (8.8 for μ and 27 for δ). The reasons for this are unclear, but perhaps are related to efficacy. On the other hand, the two other extended aromatic modifications in position 4 (**4** and **5**, Table 2) resulted in higher potencies in both the μ and δ assay systems, with 5 times greater potency in the GPI assay, and 1.6 times greater potency in the MVD (δ) for the 1-naphthylalanine modification. The biological activities at both μ and δ assays were similar to the native Biphalin when the pentafluorophenylalanine analogues was examined.

Previous thermodynamic studies have shown that Biphalin can cross phospholipid bilayer membrane with a higher partition coefficient (1.15 x $10^4$) than DPDPE (3.65 x $10^3$).[26] The 4,4'-replacement with (2*S*,3*R*)β-methylphenylalanine resulted in a 2.4 times higher partition coefficient than Biphalin itself. It was suggested that the interaction of Biphalin with the bilayer membrane involved a conformational change that allowed formation of intramolecular hydrogen bonds and aromatic ring pair interaction[26] (which also has been observed in recent unpublished NMR structure analysis in dimethyl sulfoxide solution). (2*S*,3*R*)β-Methylphenylalanine substitution might provide a favorable conformational constraints for diffusion through membranes. The increased hydrophobicity from introducing an extra methyl group into the β-position of phenylalanine side-chain also may be an important factor which is responsible for the enhanced partition coefficient across phospholipid bilayers. Membrane permeability studies currently are in progress with the other four unusual amino acid modified Biphalin analogs.

In summary, the 4,4'-positions have been further confirmed as important for Biphalin molecular design. The asymmetric (2*S*,3*R*) β-methylphenylalanine modification in the 4,4' positions provided the

highest μ opioid binding selectivity, and it is among the most selective Biphalin analogues designed so far. This modification also has resulted in greater ability to cross phospholipid bilayer membranes. In addition, the 1-naphthylalanine modification resulted in both greater binding selectivity and improved potency for the μ-opioid receptor.

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