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Research Article

The Biological Consequences of Replacing D-Ala in Biphalin with Amphiphilic α-Alkylserines

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Biphalin, a synthetic opioid peptide with a broad affinity for all opioid receptors (δ, μ, and κ) and high antinociceptive activity, has been under extensive study as a potential analgesic drug. This study presents the synthesis and biological properties of four new analogues of biphalin containing amphiphilic α-alkylserines in position 2 and 2′. The incorporation of bulky α,α-disubstituted amino acids in the peptide chain using standard peptide chemistry is often unsuccessful. We synthesized depsipeptides, and then, the desired peptides were obtained by internal *O,N*-migration of the acyl residue from the hydroxyl to the amino group under mild basic conditions. The potency and selectivity of the new analogues were evaluated by a competitive receptor-binding assay in the rat brain using [^{3}H] DAMGO (a μ ligand) and [^{3}H]DELT (a δ ligand). Their binding affinity is strongly dependent on the chirality of α-alkylserine, as analogues containing (*R*)-α-alkylserines displayed higher μ receptor affinity and selectivity than those incorporating the (*S*)-isomers.

Key words: amphiphilic amino acids, biphalin analogues, *O, N*-migration, *O*-acyl isopeptides, opioid activities, opioid peptides, α-alkylserines

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Biphalin (1), a dimeric enkephalin analogue in which tetrapeptide pharmacophores (Tyr-D-Ala-Gly-Phe) are connected 'tail to tail' by a hydrazine bridge, exhibits unique properties. It is 257- and 6.7-fold more potent than morphine (a reference μ agonist) and etorphine (an ultrapotent opioid agonist), respectively, in eliciting antinociception when administrated intracerebroventricularly (2) and leads to lesser physical dependence than morphine (3). The uniquely high potencies in both *in vitro* and *in vivo* tests of this bivalent ligand qualify it as a potent new analgesic drug (4,5). Its high analgesic activity may be related to synergic interaction with all three types of opioid receptors: μ, δ, and κ. It has been found that biphalin acts as an immunomodulator by stimulating human T cell proliferation, natural killer (NK) cell cytotoxicity *in vitro*, and interleukin-2 (IL-2) production (6). Analogues of biphalin have been studied extensively since their discovery by Lipkowski *et al.* (1) with a view to developing effective, non-addictive analgesics for the treatment pain in humans (7 and references cited therein). Previous research has explored, among others, the role of the hydrazine linker (8,9), the role of amino acid residues in positions 4, 4′ and 3, 3′ (10,11), and non-hydrazine linkers in conjunction with a modification in 4, 4′ position (12).

Biphalin remains an object of chemical modifications aimed at further improving its activity and enzymatic stability. Strategies for achieving analogs resistant to enzymatic hydrolyses included cyclization (13) and incorporation of non-coded amino acids (14). Because incorporation of amphiphilic α-alkylserines in position 2 of Leu-enkephalin led to active and long-acting analogues (15,16), our research team synthesized analogues of biphalin containing amphiphilic (*R*) or (*S*)-α-methyl and α-*iso*-propylserine in position 2, 2′ (Figure 1). We investigated the effect of substitution of D-alanine with α-alkylserines in position 2, 2′ in biphalin on opioid receptor affinities.

Experimental Section

Chemistry

All commercial reagents and solvents were used as received without further purification. The reactions were monitored and the R_f values determined using analytical thin layer chromatography (TLC) with Merck silica gel 60 and F-254 precoated plates (0.25 mm thickness). The spots on TLC plates were visualized using ultraviolet light (254 nm) and ninhydrin solution in n-butanol:acetic acid (9:1v/v) followed by heating on a hot plate.

Flash column chromatography was performed with Merck silica gel 60 (230–400 mesh). The final products were purified by RP-HPLC on Thermo Separation Products Spectra System P4000 (Thermo Separation Products, Fremont, CA, USA) (detection at 220 nm) using a Grace Smart RP C18, 5 μ, 250 mm × 4.6 mm column, HPLC solvent A

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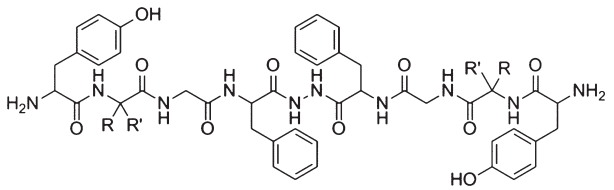


R=H, R' =CH_3 biphalin
R=CH_3, R'=CH_2OH **7a**,**7b**
R=$CH(CH_3)_2$, R' =CH_2OH **7c**,**7d**

Figure 1: Structure of biphalin and its analogues.

0.05% TFA in water, solvent B 0.038% TFA in acetonitrile: water (9:1v/v), gradient 10–50% B in A over 20 min, flow rate 1.0 mL/min.

^{1}H NMR spectra were recorded on a Bruker DPX 250 spectrometer (Bruker Biospin GMBH, Rheinstetten, Germany). Proton chemical shifts are reported in ppm (δ) relative to internal tetramethylsilane (TMS, δ: 0.00). Data are reported as follows: chemical shift {multiplicity [singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m)], coupling constants [Hz], integration}.

ESI-LC-MS was recorded on a Bruker amaZon speed ETD trap, with an ESI ion source, positive ion polarity, a maximum resolution mass range, and a 50–2000 m/z range.

General procedure for synthesis of 2

To a stirred solution of optically pure Boc-α-alkylserine (1 eq.) in DCM, TBTU (1 eq.), and DIPEA (2 eq.) were added. After stirring the mixture for 10 min, *p*-toluenesulfonate salt of glycine benzyl ester (1 eq.) was added. Then, the reaction mixture was stirred at room temperature for 24 h. The solvent was evaporated under reduced pressure; the residue was diluted with ethyl acetate and washed with three portions of 1 N $NaHSO_4$, 5% $NaHCO_3$, and brine, dried with magnesium sulfate, and concentrated under vacuum. Purification by flash chromatography (chloroform: methanol 95:5v/v) afforded the desired esters as pale yellow oils.

Boc-MeSer-Gly-OBzl (**2a**, **2b**) Yields: 93% (for (*R*)MeSer), 91% (for (*S*)MeSer), R_f = 0.7 (chloroform:methanol 9:1v/v); ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 1.43 (s, 9H, Boc); 1.44 (s, 3H, CH_3); 3.57–4.29 (m, 4H, CH_2); 5.17 (s, 2H, CH_2); 7.32–7.39 (m, 5H, Ar).

Boc-*i*PrSer-Gly-OBzl (**2c**, **2d**) Yields: 92% (for (*R*)HmVal), 87% (for (*S*)HmVal), R_f = 0.7 (chloroform:methanol 9:1v/v); ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 0.88–0.95 (m, 6H, CH_3); 1.44 (s, 9H, Boc); 1.69–1.75 (m, 1H, CH); 3.40–4.05 (m, 4H, CH_2); 5.17 (s, 2H, CH_2); 7.35–7.38 (m, 5H, Ar).

General procedure for synthesis of 3

To a stirred solution of *N*,*O*-diBocTyr (1 eq.) in DCM, TBTU (1 eq.), DIPEA (1 eq.), and DMAP (0.1 eq.) were added. After stirring the mixture for 10 min, dipeptide **2** (1 eq.) was added. Then, the reaction mixture was stirred at room temperature for 24 h. The solvent was evaporated under reduced pressure; the residue was diluted with ethyl acetate and washed with three portions of 1 N $NaHSO_4$, 5% $NaHCO_3$, and brine, dried with magnesium sulfate, and concentrated under vacuum. Purification by flash chromatography (chloroform:methanol 97:3v/v) afforded the desired *O*-acyl isopeptides as pale yellow oils.

Boc-MeSer(diBocTyr)-Gly-OBzl (3a, 3b) Yields: 91% (for (*R*)MeSer), 90% (for (*S*)MeSer), R_f = 0.85 (chloroform: methanol 9:1v/v), R_f = 0.5 (chloroform:methanol:acetic acid 95:5:1v/v/v); ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 1.42 (s, 9H, Boc); 1.43 (s, 3H, CH_3); 1.44 (s, 9H, Boc); 1.54 (s, 9H, Boc); 3.02–3.10 (m, 2H, CH_2); 3.93–4.10 (m, 2H, CH_2); 4.33–4.65 (m, 3H, CH, CH_2); 5.17 (s, 2H, CH_2); 7.06–7.19 (m, 4H, Ar); 7.33–7.37 (m, 5H, Ar).

Boc-*i*PrSer(diBocTyr)-Gly-OBzl (3c, 3d) Yields: 86% (for (*R*)HmVal), 88% (for (*S*)HmVal); R_f = 0.85 (chloroform: methanol 9:1v/v), R_f = 0.5 (chloroform:methanol:acetic acid 95:5:1v/v/v); ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 0.95–0.98 (m, 6H, CH_3); 1.40 (s, 9H, Boc); 1.42 (s, 9H, Boc); 1.55 (s, 9H, Boc); 1.69–1.76 (m, 1H, CH); 3.01–3.11 (m, 2H, CH_2); 3.95–4.21 (m, 2H, CH_2); 4.49–4,57 (m, 1H, CH); 4.54–4.88 (m, 2H, CH_2); 5.17 (s, 2H, CH_2); 7.08–7.18 (m, 4H, Ar); 7.32–7.38 (m, 5H, Ar).

General procedure for synthesis of 4

To a stirred solution of **3** in AcOH, Pd/C in MeOH was added. The reaction mixture was stirred under a hydrogen atmosphere at room temperature for 4 h. The catalyst was filtered off through Celite, and the solvent was evaporated under reduced pressure. Purification by flash chromatography (chloroform:methanol 95:5v/v) afforded the desired *O*-acyl isopeptides as pale yellow oils.

Boc-MeSer(diBocTyr)-Gly-OH (**4a**, **4b**) Yields: 84% (for (*R*)-MeSer), 86% (for (*S*)-MeSer), R_f = 0.21 (chloroform: methanol:acetic acid 95:5:1v/v/v); ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 1.42 (s, 9H, Boc); 1.43 (s, 3H, CH_3); 1.44 (s, 9H, Boc); 1.55 (s, 9H, Boc); 3.04–3.09 (m, 2H, CH_2); 3.95–4.12 (bs, 2H, CH_2); 4.34–4.61 (m, 3H, CH, CH_2); 7.08–7.18 (m, 4H, Ar).

Boc-*i*PrSer(diBocTyr)-Gly-OBzl (**4c**, **4d**) Yields: 82% (for (*R*)-HmVal), 79% (for (*S*)-HmVal), R_f = 0.21 (chloroform: methanol:acetic acid 95:5:1v/v/v); ^{1}H NMR ($CDCl_3$, 250 MHz) δ(ppm): 0.98–1.02 (m, 6H, CH_3); 1.41 (s, 9H, Boc); 1.43 (s, 9H, Boc); 1.55 (s, 9H, Boc); 1.69–1.76 (m, 1H, CH); 2.97–3.10 (m, 2H, CH_2); 3.92–4.19 (m, 3H, CH, CH_2); 4.47–4.63 (m, 2H, CH_2); 7.08–7.19 (m, 4H, Ar).

Synthesis of (2HClxPhe-NH-)$_2$ (5)

To a stirred solution of *N*-Boc-Phe (2 eq.) in DCM, TBTU (2 eq.), HOBt (2 eq.), and DIPEA (6 eq.) were added. After stirring the mixture for 10 min, hydrazine dihydrochloride (1 eq.) was added. Then, the reaction mixture was stirred at room temperature for 48 h. The solvent was evaporated under reduced pressure; the residue was precipitated out by the addition of ethyl acetate, filtered, washed with ethyl acetate and distilled water, and dried. Yield 65%, R_f = 0.5 (chloroform:methanol 95:5v/v) m.p. 152–154 °C; ^{1}H NMR ($CDCl_3$, 250 MHz) δ (ppm): 1.34 (s, 18H, Boc); 3.03–3.20 (m, 4H, CH_2); 5.04 (bs, 2H, CH); 6.07 (bs, 2H, NH); 7.16–7.23 (m, 10H, Ar); 10.62 (bs, 2H, NH-NH).

(*N*-Boc-Phe-NH-)$_2$ was deprotected by 2 N HCl in ethyl acetate at room temperature for 2 h. Then, ethyl ether was added to the reaction mixture. Precipitated crystals were filtered off and washed with ethyl ether and used for the next step without further purification. Yield 99%, R_f = 0.68 (*n*-butanol:acetic acid:water 4:1:1v/v/v) m.p. 230–232 °C.

General procedure for synthesis of 7 (Tyr-α-alkylSer-Gly-Phe-NH-)$_2$

To a stirred solution of **4** (2 eq.) in DCM, HATU (2 eq.), HOAt (2 eq.), and NMM (6 eq.) were added. After stirring the mixture for 10 min, **5** (1 eq.) was added. Then, the reaction mixture was stirred at room temperature for 48 h. The solvent was evaporated under reduced pressure; the residue was diluted with ethyl acetate and washed with three portions of 1 N $NaHSO_4$, 5% $NaHCO_3$, and brine, dried with magnesium sulfate, and concentrated under vacuum. Purification by flash chromatography (chloroform: methanol 97:3v/v), as needed, afforded the desired peptides **6** as pale yellow oils. The products were deprotected by 90% TFA_{aq} at room temperature for 2 h. The mixture was then evaporated under reduced pressure. TFA salts were dissolved in 5% $NaHCO_3$ and stirred overnight at room temperature. After lyophilization, the peptides were purified by RP-HPLC (10–50% B, 20 min) to obtain the pure products **7**.

[Tyr-(*R*)-MeSer-Gly-Phe-NH-]$_2$ (**7a**) Yield 63%, R_f = 0.88 (n-butanol:acetic acid:ethanol:water 1:1:1:1v/v/v/v), R_f = 0.57 (*n*-butanol:acetic acid:water 4:1:1v/v/v), formula $C_{48}H_{60}N_{10}O_{12}$, m/z (ESI) calc. 969.05, m/z (ESI) found 969.50, RP-HPLC: t_R 13.42 min, purity >97%.

[Tyr-(*S*)-MeSer-Gly-Phe-NH-]$_2$ (**7b**) Yield 67%, R_f = 0.88 (n-butanol:acetic acid:ethanol:water 1:1:1:1v/v/v/v), R_f = 0.57 (*n*-butanol:acetic acid:water 4:1:1v/v/v), formula $C_{48}H_{60}N_{10}O_{12}$, m/z (ESI) calc. 969.05, m/z (ESI) found 969.50, RP-HPLC: t_R 13.85 min; purity >99%.

[Tyr-(*R*)-*i*PrSer-Gly-Phe-NH-]$_2$ (**7c**) Yield 66%, R_f = 0.88 (*n*-butanol:acetic acid:ethanol:water 1:1:1:1v/v/v/v), R_f = 0.57 (*n*-butanol:acetic acid:water 4:1:1v/v/v), formula $C_{52}H_{64}N_{10}O_{12}$, m/z (ESI) calc. 1025.16, m/z (ESI) found 1025.41, RP-HPLC: t_R 13.51 min; purity >96%.

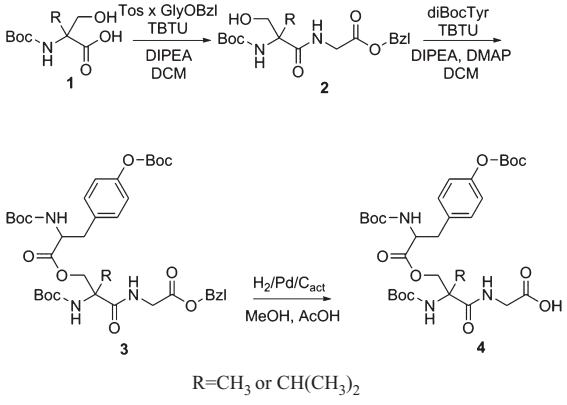
[Tyr-(*S*)-*i*PrSer-Gly-Phe-NH-]$_2$ (**7d**) Yield 62%, R_f = 0.88 (*n*-butanol:acetic acid:ethanol:water 1:1:1:1v/v/v/v), R_f = 0.57 (*n*-butanol:acetic acid:water 4:1:1v/v/v), formula $C_{52}H_{64}N_{10}O_{12}$, m/z (ESI) calc. 1025.16, m/z (ESI) found 1025.41, RP-HPLC: t_R 13, 69 min; purity >97%.

Results

Chemistry

Racemic α-alkylserines were synthesized by selective α-hydroxymethylation of the respective *N*-benzoyl-Ala and *N*-benzoyl-Val oxazolones (17). Hydrolysis of benzoylamino-5-alkyl-4-oxo-1,3-dioxanes led to racemic α-methyl(*iso*-propyl)serine. *N*-Boc-(*R*,*S*)-α-alkylserines **1** were synthesized with high yield from the potassium salt of (*R*,*S*)-α-alkylserines and Boc_2O in boiling methanol in the presence of triethylamine as a base (18) and were resolved by fractional crystallization of their diastereoisomeric salt with (-)-ephedrine. The specific rotation obtained by *N*-Boc-amino acids was consistent with that described in the literature (19).

The incorporation of bulky α,α-disubstituted amino acids in the peptide chain using standard peptide chemistry is often unsuccessful. Reagents for 'difficult' coupling, such as HATU (20) or COMU (21), proved to be efficient for the synthesis of the peptide bond, especially with the participation of the carboxyl group of α,α-disubstituted amino acids, but the amino function of these amino acids is less reactive. In the case of α-alkylserines, a free hydroxyl group can be acylated to a respective depsipeptide in which the ester bond can be easily converted into a peptide bond. In that case, the method proceeding *via* *O*-acyl isopeptides (22,23) may be the method of choice. This convenient method requires no special building blocks, is advantageous in decreasing side reactions during synthesis and in increasing the solubility of peptides during HPLC purification, and leads to higher yields of difficult sequence-con-



Scheme 1: Synthesis of protected depsipeptides.

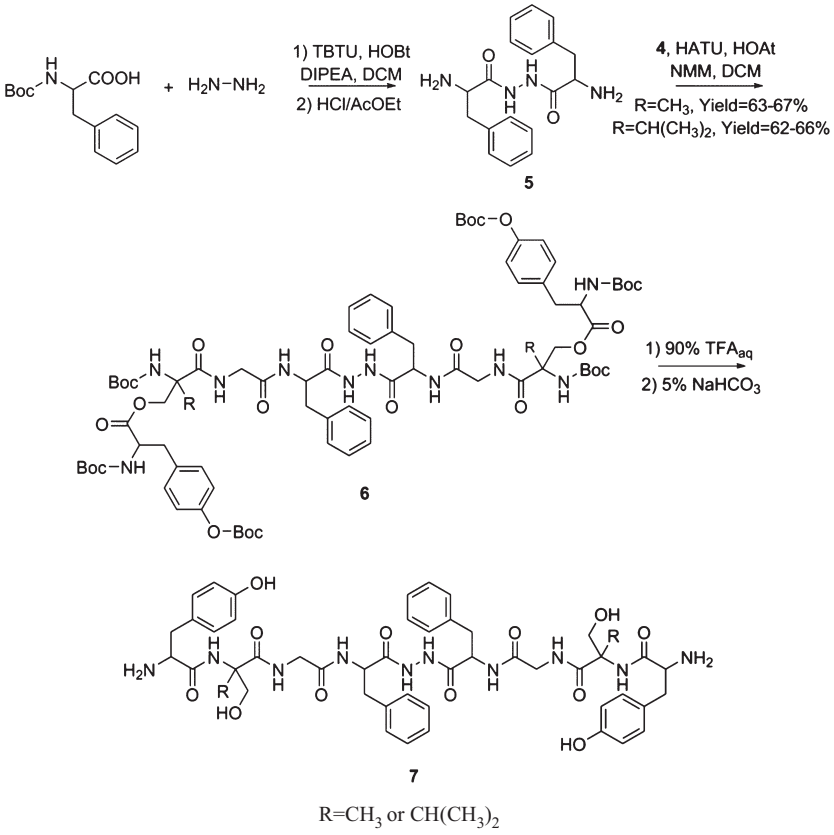
taining peptides. To obtain the target peptides, we used a (2 × 3) + 2 convergent synthesis strategy. The protected depsitripeptides **4** containing α-alkylserines in position 2 were prepared by solution techniques (Scheme 1).

The final step of the synthesis included acylation of the amino groups in the symmetric dihydrazide of phenylalanine **5** by *N*,*O*-protected tripeptides **4**. The obtained depsipeptides **6** were deprotected with 90% TFA and after an efficient internal *O*,*N*-migration of the acyl residue from the hydroxyl to the amino group under mild basic conditions; the desired analogues **7** were obtained (Scheme 2).

The crude peptides were purified by RP-HPLC. The purity of the final TFA salts was assessed by analytical HPLC (purity >96%–99%) and ESI-MS.

Molecular modeling

To investigate structure–activity relationships, the previously proposed computer-assisted intuitional analysis (CAIA) (24) was employed. SYBYL (TriposAssociates, St Louis, MO, USA) software was used for molecular modeling. Full geometry optimization was performed with an AMBER (25) force field using the SYBYL program module MAXI-MIN. The initial conformations containing α,α-disubstituted amino acid residues were constructed by replacing the respective amino acid of deltorphin with an α-aminoisobutyric acid residue, followed by energy minimization. Next, respective side-chain substitutions were introduced to form the final analogues with appropriate stereochemistry. The representative structure of each analogue that has been used in structure–activity relationship (SAR) analysis has been intuitively and arbitrarily selected from low-energy populations of



Scheme 2: Synthesis of biphalin analogues containing α-alkylserines *via* *O*,*N*-migration.

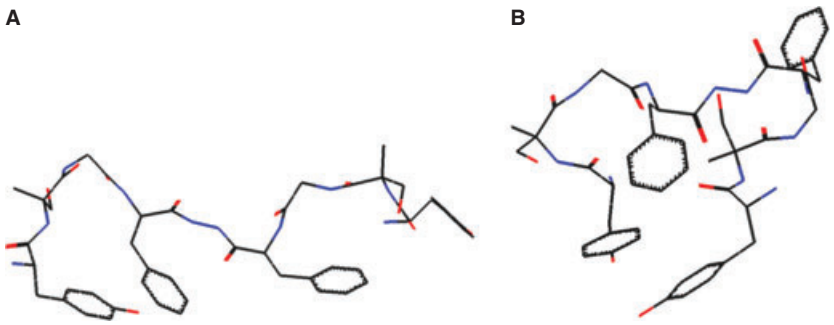


Figure 2: Extended (A) and compact (B) structure of [Tyr-(*R*)-MeSer-Gly-Phe-NH-]2.

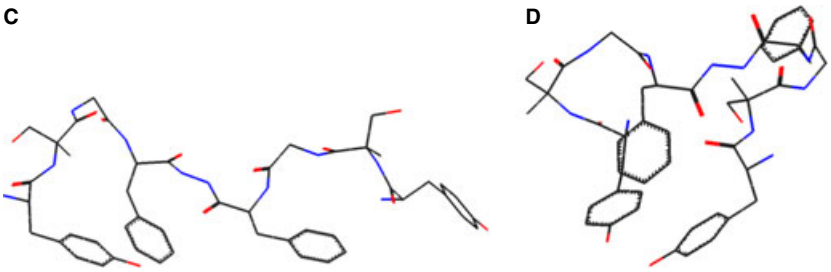


Figure 3: Extended (C) and compact (D) structure of [Tyr-(*S*)-MeSer-Gly-Phe-NH-]2.

conformers of side-chain rotamers. The selected conformers of [Tyr-(*R*)-MeSer-Gly-Phe-NH-]$_2$ and [Tyr-(*S*)-MeSer-Gly-Phe-NH-]$_2$ are given in Figures 2 and 3.

Receptor-binding activity

Receptor-binding assays were performed as described previously (19). Rat membrane preparation followed the procedure described by Misicka *et al.* (26). The radioligand receptor-binding protocol was based on a study performed by and Fichna *et al.* (27) with some modifications. The modification included different incubation time (60 min. *vs.* 120 min), bacitracin concentration (30 μg/mL vs. 50 μg/mL), and radioligand choice. The modifications were implemented in order to obtain optimal binding conditions. Binding affinities for μ and δ opioid receptors were determined by displacing [^{3}H]-DAMGO and [^{3}H]-DELT, respectively, from adult male Wistar rat brain membrane binding sites. Binding curves were fitted using nonlinear regression. Compound potency was expressed as IC_{50} values (Table 1).

Discussion

The general strategy for the synthesis of biphalin analogues by (2 × 3) + 2 fragment coupling was similar to the procedure originally described by Lipkowski *et al.* (1). In the synthesis of tripeptide-containing α-alkylserines, we used a more efficient method *via O*-acyl isopeptides.

The incorporation of the α-alkylserine residue in the peptide chain is difficult, because the sterically hindered α,α -disubstituted residues inhibit efficient peptide bond formation.

A much better yield can be obtained in the case of acylation of the free hydroxyl group in α-alkylserine. After the final deprotection, an internal *O,N*-migration of the acyl residue from the hydroxyl to the amino group under mild basic conditions leads to obtaining the desired analogues **7** in good yields.

Opioid-binding affinities at the rat opioid receptor were determined by competition analysis against [^{3}H]DAMGO (μ) and [^{3}H]DELT (δ). Our studies have shown that the replacement of (*R*)-Ala^2 with amphiphilic (*R*)MeSer^2, which is a chimera of (*S*)-alanine and (*R*)-serine (**7a**), slightly decreases affinity to μ opioid receptors, and significantly (21.5-fold) reduces that to δ opioid receptors, giving the most active and μ selective analogue in this series. Selectivity is similar to that observed for enkephalin analogues (16). On the other hand, the topographical location of the hydroxymethyl group in the (*R*)-MeSer of the tetrapeptide Tyr-(*R*)-MeSer-Gly-Phe is related to the hydroxymethyl group of (*R*)-serine in the DSLET δ-selective ligand (29). Surprisingly, the incorporation of (*S*)-MeSer, a chimera of (*R*)-alanine (D-Ala), and (*S*)-serine (L-Ser) (**7b**) clearly reduces biological activity at both receptors, but selectivity is comparable to biphalin. Binding to μ opioid receptors strongly depends on the topographical location of the hydroxymethyl group. The relative position of the aromatic ring seems to be an important feature in explaining the affinity of biphalin and its analogs to opioid receptors (30,31). The presence of two residues of amphiphilic α,α-disubstituted amino acids gives preference to a conformation binding to μ receptors.

The same trend was observed in analogues containing α-*iso*-propylserines. The peptide with (*R*)-*i*PrSer (a chimera of (*S*)-valine and (*R*)-serine) in position 2,2′ (**7c**) is more active and δ-selective than the analogue containing (*S*)-*i*PrSer (**7d**).

It is generally accepted that the (*R*)-amino acid (D-amino acid) residue in position 2 of opioid peptide analogues has dual functions. First, amino acid residues with (*R*)-chirality strongly

Table 1: Binding affinity and selectivity of biphalin analogues **7a**–**7d**

Peptide	IC_{50} ± SEM (nM) μ[a]	δ[b]	$IC^{\sigma}_{50}/IC^{\mu}_{50}$
[Tyr-D-Ala-Gly-Phe-NH]$_2$[c]	1.4 ± 0.4	2.6 ± 0.3	1.86
[Tyr-(*R*)-MeSer-Gly-Phe-NH-]$_2$ **7a**	4.88 ± 0.27	55 ± 2.27	11.3
[Tyr-(*S*)-MeSer-Gly-Phe-NH-]$_2$ **7b**	972 ± 50.3	1144 ± 52.7	1.18
[Tyr-(*R*)-*i*PrSer-Gly-Phe-NH-]$_2$ **7c**	22.4 ± 0.79	141.1 ± 3.89	6.29
[Tyr-(*S*)-*i*PrSer-Gly-Phe-NH-]$_2$ **7d**	1757 ± 84.8	1870 ± 65.6	1.06
morphine[d]	3.15	221	70.2
deltorphin II[d]	1488	0.36	0.0002

[a]*versus* [^{3}H]-DAMGO, [b]*versus* [^{3}H]-DELT II, [c]ref 10, [d]ref28.

protect peptides from aminopeptidase deactivation. In the case of disubstituted amino acid residues, both isomers equally and fully protect the peptide from enzymatic degradation by aminopeptidases. However, probably even more important is the conformational induction of appropriate local conformation(s), which is preferential in terms of ligand–receptor recognition and/or interaction. In the case of introduction of α-monofunctional amino acids, the (*R*)-chirality is preferential for both lipophilic (such as (*R*)-Ala, (*R*)-Met, (*R*)-Nle, (*R*)-Leu) as well as hydrophilic ((*R*)-Ser, (*R*)-Thr) amino acids.

Our studies showed that the analogues containing chimeras of (*S*)-lipophilic amino acids with (*R*)-Ser in position 2 and 2′ are more active.

On the basis of structural analysis of the molecular models of the analogues, we hypothesized that the hydroxyl group of the amino acid in position 2 and the amino group of the *N*-terminal tyrosine may interact and in consequence stabilize the appropriate topographical location of the tyramine moiety. A similar type of tyramine stabilization through interaction between the *N*-terminal amino group and the functional group of amino acids in position 4 has been proposed for endogenous dynorphins (26).

Conclusion

In the synthesis of biphalin analogues containing α-alkylserines, we used a more efficient method *via* *O*-acyl isopeptides. Acylation of the free hydroxyl group in α-alkylserine and after the final deprotection, an internal *O*,*N*-migration of the acyl residue from the hydroxyl to the amino group under mild basic conditions leads to obtaining the desired peptides in good yields.

The reported results indicate that the incorporation of amphiphilic α,α-disubstituted amino acid residues in position 2,2′ in biphalin gives active and selective analogues. Their binding affinity is strongly dependent on the chirality of α-alkylserine. The introduction of the (*R*)-α-alkylserine residue, the chirality of which corresponds to (*S*)-alanine or (*S*)-valine and (*R*)-serine, resulted in more active analogues **7a** and **7c**.

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