New Dimeric Tetrapeptide Enkephalin Analogues

Hydrophilic Spacer Length and Configuration Affects Potency and Receptor Selectivity

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Three new bivalent opioid peptide analogues, 1,3-di-(tyrosyl-D-alanyl-glycyl-phenylalanylamido)-2-propanol, 1,4-di-(tyrosyl-D-alanyl-glycyl-phenylalanylamido)-(2 *R*,3 *S*)-butanediol and 1,4-di-(tyrosyl-D-alanyl-glycyl-phenylalanylamido)-(2 *R*,3 *R*)-butanediol, were synthesized and tested *in vitro* for μ, δ and $\varkappa$ receptor affinities. They were found to have potent opioid receptor binding activity. The (2 *S*,3 *S*)-butanediol bridge configuration yielded selectivity and high potency for μ and $\varkappa$ receptors, while the (2 *R*,3 *R*)-butanediol bridge configuration yielded high potency and selectivity for δ receptors. It thus appears that changes in the length and configuration of the polyhydroxyl bridge in dimeric enkephalin analogues can produce a shift in receptor selectivity profiles and therefore suggest the possibility of developing more selective drugs.

Introduction

One of the promising possibility of developing new opioid analogues is the synthesis of compounds containing two pharmacophores in one molecule [1–7]. Analogues called dimeric [4, 5], double [3] or bivalent [8] enkephalins which contain bridges of various type and length between two active peptide fragments belong to this class of compounds. The nature of a bridge can alter, both, biological activity and selectivity toward opioid receptors of an analogue due to increase of enzymatic resistance and possibility of simultaneous interaction with two receptor sites.

Shimohigashi *et al.* [4, 5] have synthesized dimeric analogues of enkephalins in which two peptide fragments were connected at the C-terminus by α,ω-diaminoalkanes of variable length. The analogues exhibited generally high selectivity for δ receptors, and the authors suggested that such dimers could serve as bivalent ligands binding simultaneously to two distinct but closely clustered δ receptors. Lipkowski *et al.* [3, 9] have shown that even a shorter bivalent enkephalin analogue with a dihydrazide bond possessed relatively high activity and also δ receptor selectivity.

Recently, in an effort to reduce the conformational flexibility and to obtain more selective active compounds, a number of laboratories [10–12] have developed glycosylated enkephalin analogues which contained a sugar moiety linked to a peptide pharmocophore through various chemical bonds and showed high biological activities.

The above observations encouraged us [13] to synthesize two dimeric enkephalin analogues with hydrophilic bridges derived from 1,4-diamino-1,4-dideoxy- and 1,6-diamino-1,6-dideoxyalditols bearing two and four vicinal hydroxyl groups, respectively. These closely related analogues expressed comparable affinity for δ receptors but displayed significant differences in binding affinity for μ and $\varkappa$ receptors. The shorter spacer resulted in analogue with selectivity and high affinity for μ and $\varkappa$ receptors.

In the present study we report the syntheses of three new analogues within this series. The short spacers having one or two hydroxyl groups of various configuration have been used for bridging two peptide pharmacophores. The effect of length as well as configuration of a spacer on selectivity of a bivalent opioid ligand was of interest in this study.

Abbreviations: DCC, N,N′-dicyclohexylcarbodiimide; DCU, N,N′-dicyclohexylurea; BOC, *tert*-butyloxycarbonyl; ClCOO^{i}Bu, isobutyl chloroformate; DADLE, [D-Ala^2,D-Leu^5]enkephalin; DMF, N,N-dimethylformamide; HOBt, 1-hydroxybenzotriazole, NMM, N-methylmorpholine.

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Experimental

Chemical synthesis

Thin layer chromatography (TLC) was performed on silica gel (Kieselgel G, Merck) using the following solvent systems (v/v): (A) isopropanol : triethylamine : water = 7 : 1 : 2, (B) *n*-butanol : acetic acid : water = 4 : 1 : 2 (lower phase), (C) chloroform : methanol : benzene : 6.5 N $NH_3aq.$ = 6 : 5 : 4 : 1.

The ^{13}C NMR spectra were obtained with a Jeol FX 90 Q spectrometer. The liquid matrix secondary ions mass spectrometry (LSIMS) was performed on an AMD-604 Intectra GmbH spectrometer. Analytical HPLC analyses were performed on a Spectra-Physics SP 8800 liquid chromatograph, utilizing an analytical Supelco LC-18-T (25 cm, 5 μm, with a 2 cm guard) reversed phase column. The solvent system was buffer A (0.01 M KH_2PO_4, pH 3.0) and buffer B (50% buffer A/50% acetonitrile, v/v). The linear gradient was 40 – 100% B in 20 min, the flow rate 2.0 ml/min, and monitoring was performed at 210 nm. Semipreparative HPLC was performed on a Supelco LC-18-DB reverse phase column with linear gradients of buffer A (0.03 M ammonium acetate, pH 5.0) and buffer B (33% buffer A/67% acetonitrile, v/v) and monitoring at 230 or 260 nm.

1. 1,3-Diamino-2-propanol dihydrobromide (**1**), *1,4-diamino-(2 R,3 S)-butanediol dihydrobromide* (**2**) and *1,4-diamino-(2 R,3 R)-butanediol dihydrochloride* (**3**) were prepared according to known procedures [14 – 16] from 1,3-dichloro-2-propanol [17], 1,4-dichloro-1,4-dideoxy-*meso*-erythritol [18] and D-tartaric acid, respectively. Their ^{13}C NMR chemical shifts are given in Table I.

2. 1,3-Di-phenylalanylamido-2-propanol-dihydrochloride (**4**), *1,4-di-phenylalanylamido-(2 R,3 S)-butanediol dihydrochloride* (**5**) and *1,4-di-phenylalanylamido-(2 R,3 R)-butanediol dihydrochloride* (**6**) were prepared from appro-

Table I. ^{13}C NMR data assignments for characteristic carbon atoms in intermediates: **1 – 6, 7 a, 8 a** and **9 a** (δ in ppm).

Comp.	Solvent	Bridge carbons	Boc	Phe[a] CH	Phe[a] CH_2	Phe[a] Ar	Tyr[a] CH	Tyr[a] CH_2	Tyr[a] Ar	Ala[a] CH	Ala[a] CH_3	Gly[a] CH_2
1	D_2O	65.1 43.1	–	–	–	–	–	–	–	–	–	–
2	D_2O	69.7 42.4	–	–	–	–	–	–	–	–	–	–
3	D_2O	68.9 42.7	–	–	–	–	–	–	–	–	–	–
4	D_2O	68.6 43.2	–	55.3	37.6	134.6 130.1 129.9 128.7	–	–	–	–	–	–
5	D_2O	71.6 71.4 42.6	–	55.4	37.7	134.6 130.3 130.0 128.8	–	–	–	–	–	–
6	D_2O	70.4 42.9	–	55.3	37.7	134.7 130.2 129.9 128.8	–	–	–	–	–	–
7 a	CD_3OD	69.9 43.8[b]	157.6[c] 80.9 28.8	56.6	38.6[d]	138.5 130.3 129.5 128.8	58.1	38.6[d]	157.3[c] 131.4 127.8 116.2	[e]	17.2	43.8[b]
8 a	DMSO-d_6	71.3 71.0 [e]	155.0[f] 78.1 28.0	54.0	[e]	137.7 129.1 127.9 126.8	56.0	[e]	155.5[f] 130.0 126.1 114.6	48.1	17.9	[e]
9 a	CD_3OD	71.0 43.8[g]	157.2[h] 80.8 28.7	56.5	38.5[i]	138.4 130.3 129.4 128.7	57.9	38.5[i]	157.6[h] 131.3 127.7 116.2	[e]	17.1	44.1[g]

[a] Signals for carbonyl carbon atoms were observed but not assigned; [b,d,i] signals overlapped; [e] signal not observed because of overlapping with strong solvent lines; [c,f,g,h] assignments may be reversed.

priate diamines (**1–3**) and Boc-L-phenylalanine by the DCC-catalyzed coupling reaction in DMF as previously described [13] and subsequent deprotection with 4 N HCl in ethyl acetate at room temperature for 1 h. ^{13}C NMR chemical shifts for compounds **4–6** are shown in Table I.

3. The tripeptide Boc–Tyr–D-Ala–Gly was prepared according to Lipkowski *et al.* [3].

4. 1,3-Di-(tyrosyl-D-alanyl-glycyl-phenylalanyl-amido)-2-propanol (**7b**). A solution of Boc–Tyr–D-Ala–Gly (409 mg, 1 mmol) in DMF (2 ml) was placed on a magnetic stirrer, cooled to –20 °C and neutralized with NMM (0.11 ml, 1 mmol); then isobutyl chloroformate (0.136 ml, 1 mmol) was added. The temperature of the reaction mixture was maintained at –20 °C to – 15 °C for 15 min, and then a solution of compound **4** (0.5 mmol) with NMM (0.11 ml, 1 mmol) in DMF (5 ml) was added. The stirring was continued for 1 h at room temperature. At that time, the mixture was concentrated (1 mm Hg) and taken up with four 50 ml portions of slightly warmed ethyl acetate. The organic extracts were washed with 10% citric acid, 10% NaCl, saturated $NaHCO_3$ solution, water and dried over anhydrous Na_2SO_4. The solution was concentrated and the residue purified on a silica gel column using a gradient solvent system from 5 to 33% (v/v) methanol in chloroform. Appropriate fractions were pooled and concentrated to give compound **7a** (211 mg, 36.2%). The ^{13}C NMR spectrum (Table I) confirmed the expected structure. The Boc-protecting groups in compound **7a** (169 mg, 0.14 mmol) were removed by treatment with 4 N HCl in ethyl acetate at room temperature for 1 h. After concentration, the residue was purified on a Sephadex G-25 column using *n*-butanol – acetic acid – water (4 : 1 : 5, upper phase) as solvent. Fractions containing compound **7b** were pooled and lyophilized (85 mg, 60.7%).

Analytical HPLC of **7b** revealed single peak (97.6% by integration), retention time: 11.3 min. LSIMS[M^+] 967. TLC data: R_f(A) 0.62, R_f(B) 0.29, R_f(C) 0.65. Amino acid analysis: Gly 0.97, Ala 0.97, Tyr 0.89, Phe 1.14.

For the receptor binding assay purpose, an analytical sample of the compound **7b** was obtained by additional semipreparative HPLC purification.

5. 1,4-Di-(tyrosyl-D-alanyl-glycyl-phenylalanyl-amido)-(2 R,3 S)-butanediol (**8b**). The coupling reaction of Boc–Tyr–D-Ala–Gly (1 mmol) with compound **5** (0.5 mmol) was carried out in the same way as described above for compound **7a.** The obtained DMF solution was poured into a chilled mixture of 10% citric acid (30 ml) and 10% NaCl solution (30 ml) with stirring. The precipitation was filtered and washed successively with water, saturated $NaHCO_3$ solution and water. Recrystallization from a relatively big volume of ethyl acetate (200 ml) gave 276 mg (46.1%) of **8a.** ^{13}C NMR chemical shifts for the compound are shown in Table I. After treatment of compound **8a** (24 mg, 0.02 mmol) with 4 N HCl in ethyl acetate at room temperature for 1 h, semipreparative HPLC purification and lyophilization, compound **8b** (17 mg, 85%) was obtained in pure form.

Analytical HPLC of **8b** gave single peak (100% by integration), retention time: 11.1 min. LSIMS[M^+] 997. TLC data: R_f(A) 0.64, R_f(B) 0.32, R_f(C) 0.64. Amino acid analysis: Gly 1.06, Ala 1.03, Tyr 0.92, Phe 0.98.

6. 1,4-Di-(tyrosyl-D-alanyl-glycyl-phenylalanyl-amido)-(2 R,3 R)-butanediol (**9b**). Compound **9a** was prepared from the Boc-tripeptide (1 mmol) and compound **6** (0.5 mmol) in the same manner as described above for compound **8a.** Yield: 276 mg (46.1%). ^{13}C NMR chemical shifts for this compound are shown in Table I. Compound **9a** (24 mg, 0.02 mmol) was deprotected with 4 N HCl in ethyl acetate, and the resulted product was purified by semipreparative HPLC. After lyophilization, compound **9b** (9 mg, 45%) was obtained in pure form.

Analytical HPLC of **9b** gave single peak (97.7% by integration), retention time: 10.6 min. LSIMS[M^+] 997. TLC data: R_f(A) 0.62, R_f(B) 0.31, R_f(C) 0.61. Amino acid analysis: Gly 1.04, Ala 1.08, Tyr 0.93, Phe 0.94.

Receptor binding assays

Brains were dissected from decapitated male Hartley guinea pigs. Brain membranes preparation and receptor binding assays were performed as described previously [19, 20]. The final concentration of labelled ligands used were: 0.5 nM [^{3}H]naloxone (μ binding); 1 nM [^{3}H]DADLE in the presence of 4 nM sufentanil (δ binding); and 1 nM (–)-[^{3}H]ethylketocyclazocine (EKC) in the presence of 500 nM DADLE and 20 nM sufentanil ($\varkappa$ binding). Under these conditions, the apparent K_ds for [^{3}H]naloxone, [^{3}H]DADLE, and (–)-[^{3}H]-EKC were 0.98, 0.64 and 0.62, respectively. Binding was performed with 100 mM NaCl and bacitracin (50 μg/ml). IC_{50}s were calculated from log–logit plots. Apparent K_is were calculated from the equation, $K_i = IC_{50}/[1 + (L/K_d)]$, where L is the concentration of the radioligand and K_d is its dissociation constant. The results are shown in Table II.

Table II. Affinities of bivalent enkephalin analogues for μ, δ and $\varkappa$ opioid receptors.

Comp. No.	Compound structure	Bridge configur.	K_i [nM][a] μ	δ	$\varkappa$
10[b]	(Tyr-D-Ala-Gly-Phe-NH-)$_2$		12 ± 2	4.6 ± 0.2	270 ± 15
7b	(Tyr-D-Ala-Gly-Phe-NH-CH_2-)$_2$CHOH		62 ± 11	82 ± 7	90 ± 7
8b	(Tyr-D-Ala-Gly-Phe-NH-CH_2-CHOH-)$_2$	erythro (*R,S*)	69 ± 17	44 ± 6	137 ± 23
9b	(Tyr-D-Ala-Gly-Phe-NH-CH_2-CHOH-)$_2$	D-threo (*R,R*)	71 ± 23	10 ± 2	74 ± 8
11[c]	(Tyr-D-Ala-Gly-Phe-NH-CH_2-CHOH-)$_2$	L-threo (*S,S*)	3.2 ± 1.2	18 ± 5	1.8 ± 1

[a] Data represent mean ± S.E.M. of 3–4 experiments in duplicate; [b] reference [9]; [c] reference [13].

Results and Discussion

The syntheses of the new three dimeric enkephalin analogues **7b, 8b** and **9b** were carried out according to our previously reported strategy [13]. The synthetic route is outlined in Fig. 1. The two diamine synthons **1** and **2** were prepared by means of Gabriel reaction [14] from glycerol and *meso*-erythritol *via* their dichlorodideoxy derivatives [17, 18]. Compound **3** was synthesized from D-tartaric acid in few steps as described by Kiely *et al.* [16] for the L enantiomer. Analytical data for the diamines in appropriate forms of dihydrohalides were in good accord with the expected structures. Coupling reactions of the above diamines with N-protected phenylalanine were carried out in DMF using the DCC-HOBt method. The coupling products exhibited very low solubility in water and any inert organic solvent. Their NMR spectra were taken after deprotection of the amino groups (Table I, compounds: **4, 5** and **6**). Next, the mixed anhydride method was used for the connection of the N-Boc protected tripeptide with the phenylalanine residues. Attempts to use the DCC-HOBt coupling method on this stage have also been made, but they indicated poorer performance in relation to the mixed anhydride synthesis due to, both, yield and purity of the crude products. Target dimeric analogues were obtained after deprotection of the Boc group, chromatographic purification and lyophilization. Products **7b, 8b** and **9b** were obtained as fine, white powdery compounds with the correct amino acid analyses and LSIMS spectra. Analytical HPLC gave single peaks for these substances.

The dimeric analogues were tested for receptor binding to μ, δ and $\varkappa$ receptors in guinea pig brain homogenates by the methodology identical with that used previously for characterization of other bivalent ligands [9, 13]. The K_i values are presented in Table II. All three newly synthesized analogues exhibit different affinity profiles toward opioid

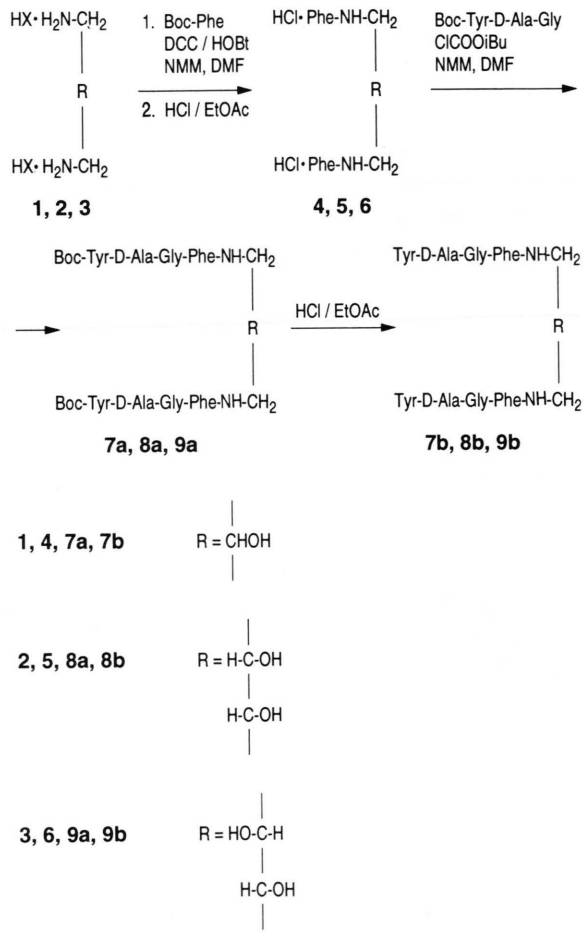

Fig. 1. Synthesis of dimeric enkephalin analogues with hydrophilic spacers.

receptors. Extension of the spacer length between the tetrapeptide pharmacophores of model compound **10** [3, 9] by three or four carbons containing one and two hydroxyl groups, respectively (compounds **7b, 8b** and **9b**), in general increased $\varkappa$ receptor affinity and decreased δ and μ receptor affinity. The *R,S* spacer configuration produced a non-selective compound (**8b**) with moderate affinity for all 3 opioid receptor types. The *R,R* spacer configuration produced a compound (**9b**) with high affinity and relative δ selectivity. The change of spacer configuration *R,R* to *S,S* (or D to L, compound **9b** and **11,** respectively) resulted in an about 20 times increase in the affinity for μ receptors and an even more spectacular increase of 40 times for $\varkappa$ receptors, while the affinity for δ receptors was not significantly affected. Thus, both, the length and configuration of the spacer are important factors in determining receptor potency and selectivity within this series.

It thus appears that the use of hydrophilic spacers creates new possibilities in the modulation of activity and selectivity of opioid peptide bivalent ligands.

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