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Synthesis of Stereoisomeric Analogues of Endomorphin-2, H-Tyr-Pro-Phe-Phe-NH_2, and Examination of Their Opioid Receptor Binding Activities and Solution Conformation

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All sixteen stereoisomeric analogues of endomorphin-2 (H-Tyr-Pro-Phe-Phe-NH_2) were synthesized by Fmoc-strategy using solid phase methods. Although synthetic endomorphin-2 exhibited similar μ- and δ-opioid receptor-binding activity to the natural compound, endomorphin-2 analogues containing D-amino acid isomers exhibited lower interaction with μ-receptors depending on the particular combination. The data clearly indicated that the three dimensional structure of endomorphin-2 with the natural L-configuration was the most suitable for binding within the μ receptor, but specific residues are important for activity. Circular dichroism studies verified that changes in chirality of amino acids in the endomorphin-2 sequence resulted in structural conformation. These alterations significantly reduced the specificity for μ-receptor-binding sites.

Key Words: **endomorphin stereoisomer; D-amino acid; μ- and δ-opioid receptors; binding activity; structure-activity relationships.**

The main classes of opioid receptors, μ, δ and κ, are located in the central nervous system and peripheral tissues of various mammalian species, such as mouse vas deferens, guinea pig ileum, rabbit jejunum, as well as in brain tissue of all vertebrates. These receptors and their endogenous ligands, the enkephalins (1), endorphins (2), dynorphins (3), and endomorphins (4) are involved in the modulation and attenuation of pain. With the exception of the μ-selective endomorphins, the mammalian endogenous ligands do not exhibit high selectivity for one receptor subtype over another. For this reason, it is interesting to design ligands that demonstrate high affinity and selectivity for a specific receptor subtype in order to study the biological function and consequences unique to each receptor and to understand the structure-activity relationships of the opioid system.

In an earlier study to improve biostability through manipulation of opioid structure, we reported data on highly novel enkephalin-derived pyrazinone-containing compounds with opioid activity (5, 6). However, in order to gain further insight on the interaction between opioid ligands with their receptors, our studies were directed to the application of D-amino acids in the new opioid peptide endomorphin-2. The rationale for the use of D-amino acids to study peptide activity is their ability to increase biological activity due to an enhanced stability against enzymic degradation (7) and the change induced in peptide conformation. This report deals with the synthesis of all sixteen stereoisomers of endomorphin-2 and compares their receptor binding activity and CD spectra.

MATERIALS AND METHODS

Peptide synthesis. Endomorphin-1 (H-Tyr-Pro-Trp-Phe-NH_2), endomorphin-2 and its diastereoisomers and H-Tyr-Pro-Phe-NH_2 were

The customary L-configuration for amino acid residues is omitted. Abbreviations used in this report for amino acids, peptides and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry,* **5,** 2485–2489 (1966); **6,** 362–364 (1966); **11,** 1726–1732 (1972). The following additional abbreviations are used: Fmoc, 9-fluorenylmethyloxycarbonyl; Bu^t, *tert*-butyl; DMF, dimethylformamide; TFA, trifluoroacetic acid; NMP, *N*-methylpyrrolidone; TFE, 2,2,2-trifluoroethanol; HOBt, 1-hydroxybenzotriazle: HBTU, 2 (1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophsphate; DIEA, *N,N′*-diisopropylethylamine; DPDPE, H-Tyr-cyclo(D-Pen-Gly-Phe-D-Pen); DAGO. H-Tyr-D-Ala-Gly-MePhe-Gly-ol.

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TABLE 1

The Retention Times, Mass Spectrometric Data, and the Amino Acid Analyses of Synthetic Peptides

Compounds	Tyr	Pro	Phe	Phe-NH_2	HPLC r.t.(min)[a]	TOF-MS[M + H]$^+$ (calcd:572.7)	Amino acid analysis		
							Tyr (1.00)	Pro (1.00)	Phe (2.00)
endomorphin-1	L	L	L-Trp	L	29.10[b]	611(calcd:611.7)[d]	0.86	0.82	1.00
endomorphin-2 [1]	L	L	L	L	36.42[b]	572[d]	0.93	1.07	2.00
[2]	D	D	D	D	16.94	572.4	0.84	1.18	2.00
[3]	L	L	D	D	18.34	572.4	0.73	1.22	2.00
[4]	D	D	L	L	18.23	572.2	0.68	1.04	2.00
[5]	D	L	L	L	17.02	572.1	0.83	1.35	2.00
[6]	L	D	D	D	17.35	572.9	0.71	1.16	2.00
[7]	L	L	L	D	18.50	572.1	0.85	1.14	2.00
[8]	D	D	D	L	18.60	572.1	0.75	1.23	2.00
[9]	L	L	D	L	20.21	572.2	0.89	1.41	2.00
[10]	D	D	L	D	20.03	572.7	0.81	0.99	2.00
[11]	L	D	L	L	19.60	572.1	0.72	1.38	2.00
[12]	D	L	D	D	20.23	572.9	0.83	1.17	2.00
[13]	D	L	L	D	18.31	572.1	0.70	0.95	2.00
[14]	L	D	D	L	18.59	572.4	0.85	1.11	2.00
[15]	D	L	D	L	19.20	572.2	0.79	1.13	2.00
[16]	L	D	L	D	19.27	572.1	0.82	1.16	2.00
[17]	L	L	L	—	16.40[c]	425.3(calcd:425.5)	0.78	1.13	1.00

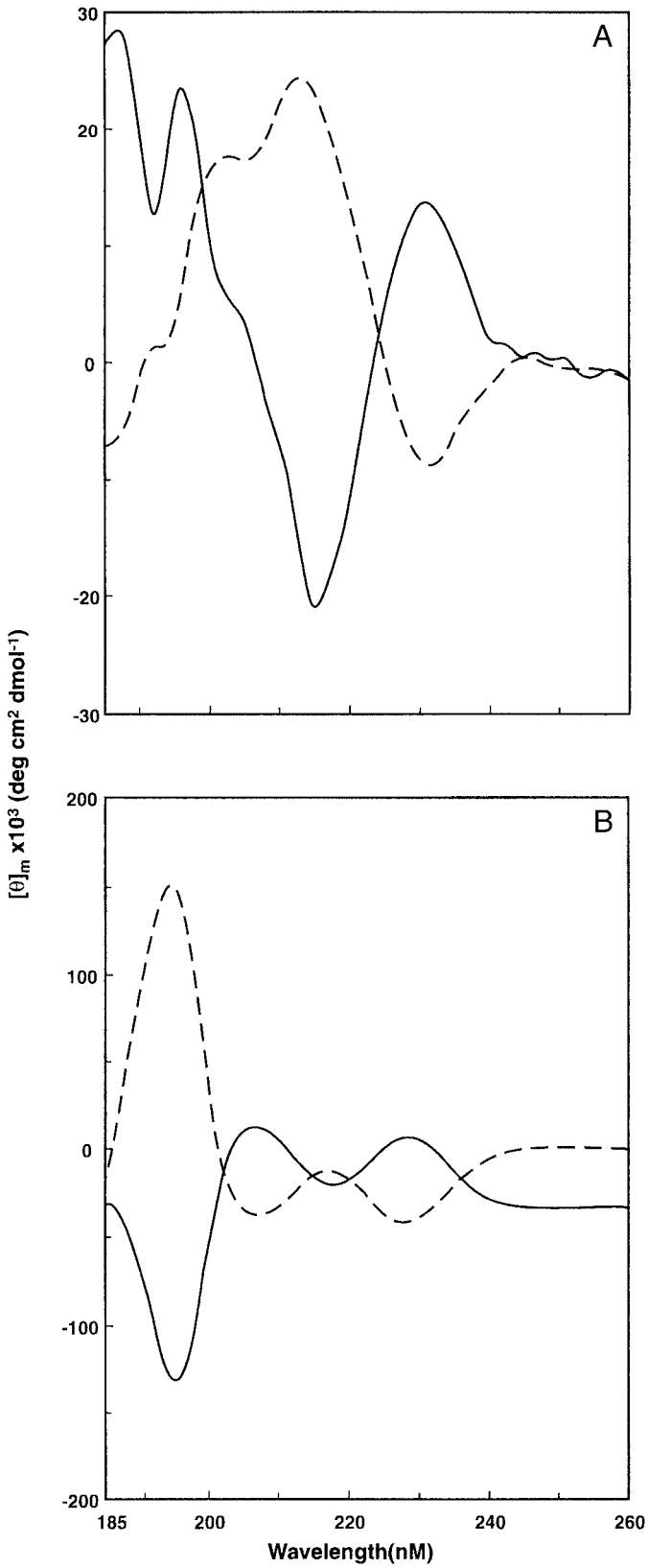
[a] HPLC A:B = 85:15 to A:B = 65:35 for 10 min, A:B = 65:35 to A:B = 45:55 for 20 min, A:B = 45:55 to A:B = 10:90 for 10 min.
[b] A:B = 85:15 for 5 min, A:B = 85:15 to A:B = 35:65 for 50 min.
[c] A:B = 85:15 for 5 min, A:B = 85:15 to A:B = 75:25 for 20 min, A:B = 75:25 to A:B = 15:85 for 15 min.
[d] FAB-MS.

synthesized by Fmoc solid-phase methods using Fmoc amide resin [4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-ethyl resin] as follows: 385 mg (0.65 mmol/g, 0.25 mmol) was used as the solid support, Fmoc-D- or L-Tyr(Bu^t)-OH (460 mg, 1.0 mmol), Fmoc-D- or L-Pro-OH (337 mg, 1.0 mmol) and Fmoc-D- or L-Phe-OH (387 mg, 1.0 mmol × 2) as the protected amino acids, and 0.45 M HBTU/HOBt/DMF (2.2 mL, 1.0 mmol), 2 M DIEA/*N*-methylpyrrolidone (NMP, 0.75 mL, 1.5 mmol) for each reaction in a peptide synthesizer ABI-433A (Applied Biosystems) according to the ABI-433A program (FastMoc 0.25 Ωmon-Prev PK). After each coupling reaction, the Fmoc group was removed by 20% piperidine/NMP for 15 min. For the final deblocking, the dried, protected peptide resin (100 mg) was suspended in TFA/H_2O (95:5, 2

TABLE 2

The Receptor Binding Data of Synthetic Peptides

Compounds	Tyr	Pro	Phe	Phe-NH_2	K_i values (nM)		
					δ	μ	$K_i\delta/K_i\mu$
endomorphin-1	L	L	L-Trp	L	1,747 ± 146	0.558 ± 0.04	3,131
endomorphin-2 [1]	L	L	L	L	7,245 ± 635	1.33 ± 0.15	5,447
[2]	D	D	D	D	16,579 ± 3,331	1,041 ± 169	98
[3]	L	L	D	D	1,249 ± 136	24.3 ± 1.8	51
[4]	D	D	L	L	19,459 ± 470	2,755 ± 640	7
[5]	D	L	L	L	4,121 ± 1,492	32.1 ± 1.5	128
[6]	L	D	D	D	13,278 ± 1,546	2,013 ± 795	6.6
[7]	L	L	L	D	8,159 ± 1,569	45.9 ± 8.6	177
[8]	D	D	D	L	7,203 ± 1,601	107.9 ± 20	67
[9]	L	L	D	L	4,230 ± 344	203.2 ± 83	21
[10]	D	D	L	D	18,624 ± 5,546	7,051 ± 791	2.6
[11]	L	D	L	L	30,641 ± 419	512.4 ± 29	60
[12]	D	L	D	D	21,264 ± 1,313	363.5 ± 89	58
[13]	D	L	L	D	4,187 ± 780	557.3 ± 174	7.5
[14]	L	D	D	L	16,662 ± 2,260	4,707 ± 714	3.5
[15]	D	L	D	L	14,584 ± 2,820	651.6 ± 37	22
[16]	L	D	L	D	26,211 ± 2,632	1,311 ± 164	20
[17]	L	L	L	—	15,899 ± 2,280	46.3 ± 3.8	343



mL) and the reaction mixture was stirred at room temperature for 2 h. The material was filtered and ether added to the filtrate to give a precipitate, which was collected by filtration and lyophilized from 1 M HCl to give the peptide hydrochloride salt. Each peptide exhibited greater than 98% purity by analytical HPLC (Waters Model 600 E) using a Cosmosil 5C18-AR column (4.6 × 250 mm) and run in the following solvents: A, 0.05% TFA in H_2O; B, 0.05% TFA in MeCN. HPLC was run under A:B (85:15) to A:B (65:35) for 10 min; A:B (65:35) to A:B (45:55) for 20 min; and A:B (45:55) to A:B (10:90) for 10 min at a flow rate of 1.0 mL/min and absorbance monitored at 220 nm.

Radioligand binding. Synaptosomal brain membrane P_2 preparations from Sprague-Dawley rats were prepared and used as the source for δ- and μ-opioid receptors (8) after the removal of endogenous opioids (8, 9). The competitive displacement assays used 5.57 nM [^{3}H]DPDPE (NEN-DuPont) and 3.5 nM [^{3}H]DAGO (Amersham) for δ and μ sites, respectively, as published (8–11). Affinity constants (K_i) were determined according to Chang and Prusoff (12).

Circular dichroism (CD) spectroscopy. CD spectra were recorded at room temperature using a JASCO J-725 spectrapolarimeter in 0.01, 0.02, and 0.1 cm cylindrical cells. Eight scans were collected for each sample over a wavelength range of 185–260 nm. The peptide solutions were prepared in neat TFE (2,2,2-trifluoroethanol) with peptide concentrations ranging from 0.9 to 1.14 mM. Band intensities are expressed as molar ellipticities, $[\theta]_M$, degrees cm^2/dmol.

RESULTS AND DISCUSSION

All the peptides exhibited a single peak on analytical HPLC and the purity was more than 98%. The analytical data for the peptides are listed in Table 1.

The receptor binding data to δ- and μ-opioid receptors are detailed in Table 2. The synthetic endomorphin-1 and -2 peptides exhibited very similar receptor affinities and selectivities as reported for the natural isolates (4). All the D-amino acid analogues of endomorphin-2 decreased their effectiveness with respect to the μ-opioid receptor; however, specific residues could be discerned as having greater importance in the interaction of the peptide within the receptor as demonstrated with deltorphin analogues (13). For example, endomorphin-2 [**1**] had a $K_i\mu$ = 1.33 nM, but its D-amino acid antipode [**2**] only exhibited a very weak interaction ($K_i\mu$ = 1041 nM) and the binding to δ receptors also decreased by more than a factor of two. The CD spectra comparing **1** and **2** verifies the change in isomeric conformation in that **2** is the negative spectra obtained with **1** as seen in Fig. 1A. Similarly, while compound **3** bound to μ receptors, its antipode [**4**] was bound 113-fold weaker. The comparison of the CD spectra between these two peptides revealed that **4** had a different conformation than **3** due to the presence of the D-amino acid isomers (Fig. 1B). Although **5** had a

FIG. 1. CD spectra of endomorphin-2 and its analogues in TFE. (A) 0.993 mM endomorphin-2 (- - -) and 1.13 mM H-D-Tyr-D-Pro-D-Phe-D-Phe-NH_2 (—). (B) 1.14 mM H-Tyr-Pro-D-Phe-D-Phe-NH_2 (- - -) and 1.07 mM H-D-Tyr-D-Pro-Phe-Phe-NH_2 (—).

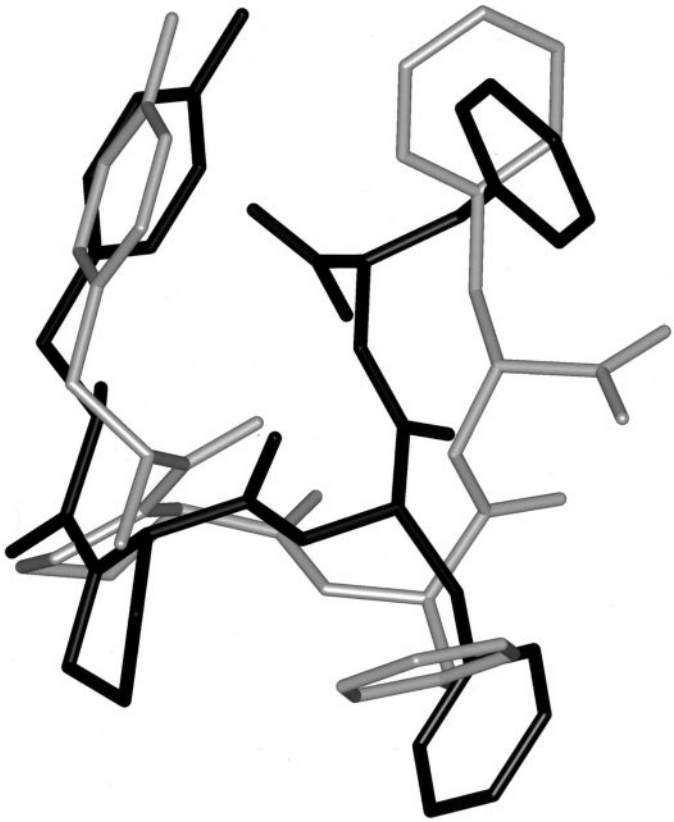


FIG. 2. Superposition of low energy structures of the biologically active endomorphin-2 (black, $K_i\mu$ = 1.33 nM) and its D-isomer antipode (grey) that is inactive ($K_i\mu$ = 1041 nM). Models were based on an ^{1}H NMR derived structure of *cis*-endomorphin-1 (15).

$K_i\mu$ similar to that of **3,** the activity of the antipode [**6**] was as drastically reduced as seen with **4** suggesting the possibility that Tyr plays a marked role in peptide recognition by the receptor (13, 14): as long as the remaining amino acids in the subsequent tripeptide contained L-isomers, the negative impact of an N-terminal D-Tyr on μ affinity was minimal. This fact is borne out by the observation that L-Tyr partially overcame the detrimental effect of the D-isomers in **6** as compared to compound **4.**

It is interesting to note that even though the presence of C-terminal L-Phe appears to optimally generate μ-receptor binding activity [**1**], the D-isomer [**7**] or its absence in a tripeptide [**17**] provided the same results. However, D-Tyr^1 [**13**] further augmented the effect of a modified configuration of Phe^4 [**9**]. These data suggest that the remaining portion of the tripeptide contained key factors for binding within the receptor pocket, including the essential Tyr residue. In fact, the reversed polarity of peptide [**8**] further demonstrated that the D-configuration in the N-terminal tripeptide was important for the binding mechanism. Of these residues, Pro^2 [**11, 12, 16**] and Phe^3 [**9**] appear to be quite essential: Their D-isomers dramatically reduced μ affinity. Furthermore the antipode of compound **9** demonstrated the requirement for the simultaneous L-configuration for both Tyr^1 and Pro^2 [**10, 14**]. The disruptive effect of the topography of the peptide by the D-enantiomers was verified by the CD spectra (Fig. 1). Low energy models of endomorphin-2 and its D-isomer antipode that were calculated based on the ^{1}H NMR structure of *cis*-endomorphin-1 (15) served to display the change in topography (Fig. 2). The superimposition of the peptides demonstrated in Fig. 2 clearly reveals the spatial orientation of the Pro residue and its potential role as a discriminatory element for μ selectivity. The D-isomeric combination at Tyr and Phe^3 [**15**] once again substantiates our observations on the importance of these residues.

In conclusion, the topographical constraints by the L-configuration in the N-terminal tripeptide moiety (H-Tyr-Pro-Phe) of endomorphin-2 appear optimal and act in a concerted manner to align the peptide within the μ receptor. Although L-Tyr remains a critical residue for the receptor binding of opioid peptides, L-Pro must be considered vital for the expression of μ-receptor selectivity for endomorphin-2. Sequential replacement by D-isomers in all 16 possible combinations, in addition to the [des-Phe^4]endomorphin-2 analogue [**17**] provides evidence that some residues play a greater role in the ligand binding mechanism which was also found to be the case for deltorphin analogues (13–16).

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