Stereospecificity of Amino Acid Side Chains in Deltorphin Defines Binding to Opioid Receptors

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A series of individual D-amino acid replacement analogues of deltorphin A, several of which were in combination with a His[4] deletion, were used to probe alterations of side-chain orientation on peptide binding parameters with rat brain opioid receptors. Peptides with D-amino acids in residues 1, 3, and 5 exhibited diminished affinities primarily for δ receptors (88–1200-fold) with selectivity decreasing by factors of 13–64-fold relative to deltorphin A ($K_i\delta$ = 0.45 nM; $K_i\mu/K_i\delta$ = 764): the aromatic side chains Tyr[1] and Phe[3], which lie in the N-terminal "message" domain and the aryl side chain of Leu[5] in the C-terminal "address" domain, appear to play essential roles in conferring high δ affinity and selectivity. Although D-His[4] only decreased δ affinity by 6-fold and selectivity by a factor of 4, His appears to be involved as an integral component of both domains: [des-His[4]]deltorphin A and [des-His[4]] analogues containing consecutive D-amino acid replacements in the remaining residues exhibited weak binding to δ receptors and poor δ selectivity. Substitution of D-Met[2] in deltorphin A by D-Ala or D-Nle decreased δ selectivities 3–6-fold through an elevation in μ affinities; however, the converse replacement, D-Met for D-Ala[2] in deltorphin B, diminished β selectivity by an order of magnitude only through the loss in δ affinity. The data show that the high δ affinity and selectivity of deltorphins correlate with and require a strict stereospecificity of the amino acid residue side chains.

Introduction[1]

Attempts to obtain analogues of opioid peptides with high affinity and selectivity for a specific class of receptor sites were a major goal in the investigations on morphinomimetic peptides for well over a decade.[2] The recent discovery of the dermorphin and deltorphin families of opioid heptapeptides from frog skin[3-5] set the stage for renewed interest in opioid peptides. The deltorphins, for example, possess very high affinity for δ receptors and unparalleled δ selectivity using both brain membrane binding assays[4-13] and pharmacological preparations from guinea pig ileum and mouse vas deferens.[3,4,9,13]

Structure–activity studies were undertaken in order to probe selected molecular features which contribute to the avidity of the deltorphins for δ receptors.[6-8,13] Those results suggested that the deltorphins, like other peptide hormones, contain "sychnologic organization";[14] i.e., two distinct, proximal regions[15] which confer specific attributes to the peptide, a N-terminal "message" domain that defines biological responsiveness and a C-terminal "address" domain that influences binding affinities[11,15] for a specific receptor type. Recent data suggested that a negative charge, embedded within the latter region of the deltorphins was primarily responsible for δ selectivity,[7,12,13] as postulated by Schwyzer on studies with enkephalins.[16] However, the combination of high δ affinity and selectivity appeared dependent not only upon the presence of an anionic group, but also residues with hydrophobic side chains within a defined sequence in the "address" domain,[7] in addition to a C-terminal amide group.[5,7,13,17,18]

This communication presents an extension of our reconnaissance into the dissection of the structure of deltorphins[7,8] in order to examine the relative orientation of the side chains of the residues which contribute to their interaction with opioid receptors. Toward that goal, each amino acid residue in deltorphin A (H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2) and deltorphin A [des-His[4]]-hexapeptide analogues was replaced with its D-enantiomer; this modification induces further alterations in the spatial orientation of side chains and presumably the conformation of the peptide backbone.[19] The importance of His[4] in deltorphin was previously recognized and considered to be involved in maintaining a solution conformation,[18] high affinity binding to the δ receptor,[12,13] and a constituent component of the "message" domain.[8] The preparation

Table I. Binding Parameters of D-Amino Acid-Substituted Deltorphin Analogues[a]

no.	peptide	$K_i\delta$, nM	$K_i\mu$, nM	$K_i\mu/K_i\delta$
1	deltorphin A	0.45 ± 0.04	343.8 ± 12.8	764
2	[D-Tyr[1]]	544.0 ± 56.9	7,410 ± 809	14
3	[D-Ala[2]]	0.36 ± 0.12	43.7 ± 8.1	121
4	[D-Nle[2]]	0.12 ± 0.02	32.6 ± 0.34	272
5	[D-Phe[3]]	39.6 ± 3.1	2,351 ± 209	59
6	[D-His[4]]	2.77 ± 0.32	492.0 ± 90.7	178
7	[D-Leu[5]]	58.5 ± 0.41	690.6 ± 32.6	12
8	[D-Met[6]]	0.96 ± 0.07	146.2 ± 13.0	152
9	[D-Asp[7]]	0.54 ± 0.08	124.5 ± 33.7	230
10	[des-His[4]]	12.6 ± 0.57	351.7 ± 40.8	28
11	[D-Tyr[1],des-His[4]]	476.5 ± 68.6	7,410 ± 296	16
12	[D-Ala[2],des-His[4]]	13.9 ± 1.8	567.1 ± 70.5	41
13	[D-Phe[3],des-His[4]]	741.5 ± 79.9	2,253 ± 59.3	3
14	[des-His[4],D-Leu[5]]	120.4 ± 24.1	330.0 ± 10.4	3
15	[des-His[4],D-Met[6]]	25.8 ± 1.18	407.1 ± 58.5	16
16	[des-His[4],D-Asp[7]]	9.46 ± 1.84	266.7 ± 36.0	28
17	deltorphin B	0.41 ± 0.09	1,280 ± 192	3,122
18	[D-Met[2]]	3.30 ± 0.51	1,047 ± 71	317

[a] The sequences of deltorphins A and B are H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2 and H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2, respectively. The inhibition constant K_i (mean, nM, ±SEM; n = 3–8) was determined using the formulation of Cheng and Prusoff:[51] $K_i = IC_{50}/(1 + L/K_d)$, where L is the concentration of radioactive ligand and K_d represents the dissociation constants for either [^{3}H]DPDPE or [^{3}H]DAGO. $K_i\mu/K_i\delta$ is defined as δ selectivity.

(1) Symbols and abbrevations used in this paper conform to the recommendations of the IUPAC-IUB Joint Commission on Nomenclature (*J. Biol. Chem.* **1972**, *247*, 977–983). The amino acids are the optically active L-isomers unless stated in the Experimental Section. Other abbreviations are as follows: Atc, 2 aminotetralin-2-carboxylic acid; Boc, *tert*-butyloxycarbonyl; Bum, *N*-(π)-*tert*-butoxymethyl; DAGO, [D-Ala[2],NMePhe[4],Gly-ol[5]]enkephalin; DIC, diisopropylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; DPDPE, cyclic [D-Pen[2,5]]enkephalin; Fmoc, [(9-fluorenylmethyl)oxy]carbonyl; OSu, *N*-succinimidoyl; TFA, trifluoroacetic acid; Trt, triphenylmethyl. Deltorphin A[7,8] is also referred to as "dermorphin gene-associated peptide",[6] "deltorphin",[4,12] and "dermenkephalin",[5,10,11,17] while [D-Ala[2]]deltorphin I and I[4] were redefined as deltorphin B and deltorphin C, respectively, for the sake of clarity.[7]

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of [des-His[4]]deltorphin A analogues provided an opportunity to assess the contribution of the C-terminal tri-

Table II. Analytical Properties of Synthetic Deltorphin Analogues[a]

peptide	TLC:[b] R_f I	R_f II	HPLC: K'[c]	melting point, °C[d]	$[\alpha]^{22}_D$[e]
1	0.45	0.38	8.75	162–164	−9.2
2	0.65	0.52	8.06	148–150	−7.5
3	0.51	0.45	7.11	141–143	−5.7
4	0.59	0.56	7.47	186–188	−6.3
5	0.61	0.54	8.33	136–138	−4.1
6	0.59	0.55	8.12	138–140	−28.7
7	0.62	0.51	8.95	145–147	−9.1
8	0.63	0.48	9.54	143–145	−5.7
9	0.61	0.47	8.53	140–142	−4.3
10	0.74	0.66	8.38	143–150	−17.9
11	0.72	0.58	7.72	171–173	−5.7
12	0.69	0.58	7.42	118–120	−3.8
13	0.75	0.66	7.72	153–155	−6.9
14	0.75	0.69	9.80	178–180	−1.7
15	0.75	0.71	8.96	160–162	−1.3
16	0.74	0.66	8.29	145–147	−2.2
17	0.62	0.45	5.37	185–187	−14.7
18	0.81	0.60	6.85	222–224	−2.3

[a] Synthetic deltorphin analogues were analyzed as follows: [b] Merck precoated 0.25-mm analytical silica gel plates $60F_{254}$. Solvent systems are I, butanol/HOAc/H_2O (3:1:1); II, butanol/HOAc/pyridine/H_2O (12:4:2.2:1.2). [c] Capacity factor (K') was determined using Spherisorb 5-ODS2 (HPLC-technology) C-18 reversed-phase column (46 X 250 mm) with a gradient consisting of two mobile phases: A, 10% (v/v) acetonitrile in 0.1% TFA, and B, 60% (v/v) acetonitrile in 0.1% TFA. A 30-min linear gradient was run from 20% to 80% B at a flow rate of 1 mL/min. [d] Melting points were determined on a Kofler apparatus and are uncorrected. [e] Optical rotations were conducted in methanol (peptides 1–17) or dimethylforamide (peptide 18) using a 10-cm pathlength cell in a Perkin-Elmer 241 polarimeter.

peptide "address" domain, Leu[5]-Met[6]-Asp-NH_2[7], on the interaction with opioid receptors as a consequence of being repositioned adjacent to the common message sequence, H-Tyr[1]-D-Xaa[2]-Phe[3]. The significance of the side chain of the D-amino acid residue at position 2 of deltorphin A and deltorphin B (H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2) was also evaluated. These studies afford important insights into the stereospecificity of selective amino acid side chains in the "message" and "address" domains which confer high δ affinity and selectivity properties to the deltorphins.

Results

Modulation of δ and μ Affinities. The systematic substitution of D- for L-amino acid isomers in deltorphin A *differentially* modified both δ and μ K_i values (Table I). Peptides containing D-enantiomers substituted at positions 1, 3, and 5 exhibited greatly diminished δ affinities; in particular, the transposition of L- to D-Tyr[1] had a major effect on both δ and μ affinities which fell over 1200- and 21-fold, respectively. Replacement analogues with D-His[4] (6) and D-Met[6] (8) exhibited moderately curtailed δ affinities, while that with D-Asp[7] (9) was comparable to deltorphin A (1). On the other hand, μ affinities primarily declined for peptides with D-amino acids at residues 1, 3, and 5, and to a lesser degree at position 4, and increased in those analogues substituted at residues 2, 6, and 7. The exchange of D-Met[2] by D-Ala[2] (3) failed to affect δ affinity, while replacement with D-Nle[2] (4) increased δ affinity 4-fold; both substitutions enhanced μ affinities 8–10-fold. In contrast, replacement of D-Ala[2] with

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D-Met^2 in deltorphin B (18) only decreased δ affinity (Table I).

Deltorphin A His^4 Deletion Analogues. Deletion of His^4 in analogues containing L- or D-amino acid substitutions (10–16) uniformly diminished high affinity δ binding by 1 to over 3 orders of magnitude; in contrast, μ affinities varied considerably and frequently in a nonparallel manner (Table I). A comparison of the D-enantiomers of Tyr^1 (11) and Phe^3 (13) revealed that they both reduced δ and μ affinities: the effect of D-Tyr^1 was essential similar in the des-His^4 analogue 11 as in the heptapeptide 2, whereas δ affinity in the [D-Phe^3,des-His^4] analogue 13 decreased over 3 orders of magnitude compared to an 88-fold decline in δ affinity in [D-Phe^3]deltorphin A (5). On the other hand, D-Leu^5 primarily affected δ affinity regardless of the presence (7) or absence of His^4 (14). It is interesting that the μ affinities in several analogues (10 and 14–16) were comparable to that of deltorphin A (1). In spite of the apparent modification of the lipophilicity of these peptides, estimated by reversed-phase HPLC (Table II), linear regression analyses proved that the K_i values for δ and μ binding sites (Table I) for the D-amino acid-containing heptapeptides and for the des-His^4 analogues versus K' (Table II) were statistically nonsignificant.

Selectivity Effects. The D-amino acid analogues of deltorphin A exhibited distinctive combinations of δ and μ affinities; the result of which was an ablation of high δ selectivity (Table I). Although the δ affinities of peptides 3, 8, and 9 were similar to that of deltorphin A (1), while that of peptide 4 was higher, their elevated μ affinities mitigated the potential for higher δ selectivities. As a class of peptides, the analogues containing a His^4 deletion exhibited considerably lower δ selectivity than the individual D-amino acid replacement analogues, except those containing D-Tyr^2 and D-Leu^5 (2 and 7).

Discussion

Our data unambiguously demonstrate that inversions of amino acid side-chain configuration result in spatial alterations which affect the abilities of deltorphin analogues to interact with opioid receptors. D-Amino acid substitution analogues of opioid peptides, such as morphiceptin,[20] β-casomorphin,[21] enkephalin,[2,22-24] and β-endorphin,[25] as well as other peptide hormones [e.g., LHRH,[26] α-melanotropin,[19,27] neuropeptide Y,[28] neurotensin,[29] somatostatin,[30] substance P,[31] and tuftsin[32]] led to the recognition of peptides with altered characteristics that conferred enhanced stabilities,[23] higher affinities,[2,20-22] greater potencies,[19,21,27] or antagonist properties.[26,31,33] However, the D-amino acid isomers in deltorphin analogues conferred lower δ selectivities due to modification in δ or μ affinities; none of these analogues exhibited a δ selectivity approaching those of deltorphins A or B. These results contrast with data obtained with D-isomer analogues of other opioid peptides, which were occasionally more selective than the parent peptide.[2,20,22,34]

The reduction in δ affinity of D-Tyr^1 and D-Phe^3 substitution analogues may reflect a disruption of the N-terminal type II β-turn,[35-37] which appears to be involved

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in binding to μ receptors,[38] and leads to a suboptimal spatial alignment of the hydroxyl group of Tyr[1]. The orientation of the plane of the aromatic ring of D-Phe[3] could possibly lead to a modification of the conformation in the N-terminal sequence:[17,18,35,37] the consequence of which could disrupt internal H-bonding of Tyr[1]-NH_3 to Asp[7],[18] or the interaction of the tyramine side chain with a possible receptor site. However, conformational restriction imposed by substitution of Atc[3] in deltorphin C led to an increase of nearly 3-fold higher bioactivity.[39] Thus, the spatial alignment of the residue at position 3 is essential for receptor recognition.

Data with des-His[4] analogues suggest a potential role for His[4] in maintaining the spatial orientation of the C-terminal sequence of deltorphin A in solution. The absence of His[4] could diminish the extent of H-bonding between Tyr[1] C=O and His[4] N—H (which, by analogy to dermorphin,[36] permits formation of a 10-membered ring) and disrupt the H-bond that could form between the His[4] imidazole ring and Asp[7].[18] The absence of the His imidazole also alters the overall spatial orientation of the peptides (10–16), while its replacement by Gly, Phe, or Tyr, which caused minor changes in δ affinities, substantially increased μ binding.[13,42] It can be postulated then that the consequences of His[4] deletion might include: (i) a repositioning of the hydrophobic core (Leu[5]-Met[6]) relative to a corresponding area in the receptor or (ii) modifying the dynamics of maintaining an optimally folded conformer.[18,37,38] However, regardless of the presence or absence of His[4], the spatial orientation of Tyr[1], Phe[3], and Leu[5] exert a major role in receptor binding. On the other hand, the remaining substitutions exhibited differential effects on δ and μ affinities and suggest that His[4] appears to function as an integral component in the preservation of the optimal spatial orientation required for binding of the peptide in the receptor.

Replacement of D-Met[2] in deltorphin A with D-Ala or D-Nle enhanced μ affinities; however, since NMR data indicate that the conformation of the side chain of Met does not differ from that of Nle,[40] the K_i values for binding are presumed to reflect differences in amino acid side-chain hydrophobicity which are reflected in the changes in K' values of peptides 3 and 4 relative to that of deltorphin A (1) (Table II). Similarly, the substitution of Met by Nle in α-MSH[19] and cholecystokinin-8[41] enhanced bioactivity. The substitution of D-Ala[2] in deltorphin A may introduce greater stability due to the interaction between the D-Ala[2] methyl group and the Phe[3] aryl ring[18,35,37] and modified the apparent lipophilicity of the peptide. That the side chain of residue 2 affects opioid affinity and selectivity was indicated by substitution of L-Met[2] in deltorphin A, which greatly reduced bioactivity and receptor binding (~1000–10000-fold).[5,6,9–11] Additional evidence on the orientation of residue 2 was also provided by data with a series of synthetic enkephalin analogues: optimal bioactivity occurred in which *gem*-dimethylethanediamine was incorporated in lieu of the second amino acid.[24]

A requirement for C-terminal hydrophobic residues in determining δ selectivity could possibly be correlated with ^{1}H NMR data which indicated the involvement of C-terminal residues in a proper conformation.[18,37] The detrimental effect of the D-Leu[5] substitution (7) may then be attributable to the misalignment of the hydrophobic side chain and the proposed peptide conformation:[18,37] the evidence of disruption in peptide conformation, or a change in the handedness of the peptide backbone,[19] is supported by the loss of δ binding by over 2 orders of magnitude (Table I). Retention of δ selectivity by a deltorphin A N-terminal pentapeptide,[13] in contrast to deltorphin A tetrapeptides which are μ selective,[5,8,11–13] indicates that δ selectivity requires the existence of a hydrophobic residue at position 5 and the presumed H-bond interactions involving C-terminal residues at positions 6 and 7, and the N-terminal sequence within the peptide.[18,37]

The marginal effect of D-Met[6] (8) on δ affinity, accompanied by a comparable increase in μ affinity, differs markedly from published observations,[11] and complements studies on analogues with substitutions at position 6;[42] however, the change in the side-chain orientation affected peptide lipophilicity (Table II). The β-carboxyl function of Asp[7] was proposed to stabilize the peptide by hydrogen bonding,[18,37] and by analogy with enkephalin,[39] through "head to tail pseudo-cyclization" by forming a salt bridge with the amino group of Tyr[1];[37] replacement with D-Asp[7] (peptide 9) had no effect on $K_i\delta$, but increased μ affinity 3-fold, which suggests that δ and μ receptor sites might select or require different peptide conformers.[38]

Modification in the spatial orientation by amino acid side chains apparently influences the amphiphilic moment of the peptide.[16] In addition to a characterization of deltorphin on the basis of the sequence determinants of "messenger" and "address" domains, a comprehensive description of their properties should incorporate lipophilicity,[16] side-chain orientation, solution conformers,[38] and internal hydrogen bonding.[18,37] The results with D-enantiomer-containing analogues of deltorphin further suggest that the proposed charge effects of the membrane and ligand for the δ selectivity may only be valid for the enkephalins.[16] The δ receptor site appears able to discriminate among the various conformers of deltorphin A: the orientation of the side chains assists in the maintenance of a preferred solution conformation through an interaction with distinct hydrophilic and hydrophobic regions.[16]

Conclusions

The sequential substitution of D-amino acid isomers in deltorphin provided evidence that the side chains of at least three residues are important and appear to be required for high δ affinity and selectivity, namely Tyr[1], Phe[3], and Leu[5]. However, the application of a His[4] deletion and des-His[4] analogues with the systematic replacement with D-amino acids provided information on the involvement of His[4] on receptor selectivity and a necessity for the proper alignment of the remaining side chains of the residues and a specific amino acid sequence in the C-terminal region. The proposal that the C-terminal tripeptide of deltorphin "strongly influences the three-dimensional

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structure of the entire molecule"[17] may be essentially correct while it may also understate the spectrum of interaction capabilities of C-terminal substituents. It appears therefore that the deltorphins, with their high δ affinities and δ selectivities are a useful group of peptides which can be applied in the design of new compounds to probe opioid receptors.

Experimental Section

General Methods for Peptide Synthesis. Deltorphins A and B and most analogues used in this study were synthesized by solid-phase methods.[7,13] The solid support, 4-(2',4'-dimethoxyphenyl)Fmoc-aminomethylphenoxy resin (0.43 mmol/g; 0.2 mmol), was obtained from Bachem (Torrance, CA), and the protected amino acids were from Bachem or Novabiochem AG (Germany). With the exception of N$^{\alpha}$-terminal N^{α}-Boc-Tyr, N^{α}-Fmoc amino acid derivatives were used in the coupling reactions; the reactive side chain of Asp was protected with *tert*-butyl ester, while His was protected with *tert*-butoxymethyl or Trt groups. Amino acids were double coupled to the growing peptide chain using preformed symmetrical anhydrides, except N^{α}-Fmoc-His(Bum)-OH and N^{α}-Fmoc-D-His(Trt)-OH, which were coupled by the DIC-DMAP procedure;[43] the N^{α}-Boc-Tyr-OH was coupled as the preformed OSu ester. Coupling was monitored with ninhydrin and by amino acid analysis of acid hydrolysates. Nascent protected peptides were cleaved from the resin by treatment with 95% TFA (10 mL/g of resin) for 3 h at 20 °C. Crude peptides were initially purified by gel filtration on Sephadex LH-20 (2.5 × 100 cm) columns eluted with methanol/HOAc/H_2O (18:2:80) or preparative HPLC; HPLC used a Waters Delta Prep 3000 with a Delta Pak C-18 300-Å, 15-μm spherical particle column (30 × 300 mm), eluted with a 30-min linear gradient from 20% mobile phase A [10% (v/v) acetonitrile in 0.1% TFA] to 80% mobile phase B [60% (v/v) acetonitrile in 0.1% TFA] at a flow rate of 30 mL/min and the effluent monitored at 220 nm. Purity was assessed by analytical HPLC[7,13] and TLC in two solvent systems; peptide homogeneity was >99%. Amino acid compositions were quantitated using a Carlo Erba 3A-29 amino acid analyzer. ^{1}H NMR spectra agreed with the amino acid sequence and composition of each analogue.

Coupling Procedures. The analytical properties of the synthetic deltorphin analogues are listed in Table II.

Deltorphin A (1). This peptide was synthesized and purified by published methods[7,13] using a solid support, 4-(2',4'-dimethoxyphenyl)Fmoc-aminomethylphenoxy resin[44] (0.43 mmol/g; 0.2 mmol) obtained from Bachem (Torrance, CA). Coupling was monitored with ninhydrin.[45]

[Des-His^4]deltorphin A (10). [Des-His^4]deltorphin A was prepared as follows: to a solution maintained at −10 °C, containing H-Leu-Met-Asp(OtBu)-NH_2 (0.108 g, 0.25 mmol), 1-ethyl-3-(dimethylamino)propylcarbodiimide[7] (50 mg, 0.26 mmol) in dry DMF (10 mL) was added *N*-hydroxybenzotriazole[46] (0.1 g, 0.75 mmol) and the tripeptide, N^{α}-Boc-Tyr-D-Met-Phe-OH (0.125 g, 0.25 mmol). The mixture was initially stirred at −10 °C for 2 h and then at room temperature overnight and poured into a solution of ethyl acetate/aqueous $NaHCO_3$/citric acid (13). Extraction and purification by gel filtration gave a protected heptapeptide (0.195 g) in 80% yield; deblocking occurred by exposure to TFA/CH_2Cl_2 (1:1, v/v) for 60 min at 0 °C. Amino acid analysis gave the following molar ratios: Tyr, 0.91; Met 1.94; Phe, 0.95; Leu, 1.0; Asp, 0.97.

[D-Tyr^1]- and [D-Tyr^1,des-His^4]deltorphin A (2 and 11, Respectively). Solid-phase synthesis was accomplished by stepwise addition of the following protected amino acids to 0.5 g of resin (0.2 mmol): N^{α}-Fmoc-Phe, N^{α}-Fmoc-Met, N^{α}-Fmoc-Leu, N^{α}-Fmoc-His(Bum), N^{α}-Fmoc-Asp(OtBu), N^{α}-Fmoc-D-Met, and N^{α}-Boc-D-Tyr. N^{α}-Fmoc-His(Bum) (0.4 mmol) was introduced by coupling with DIC (0.4 mmol) in the presence of a catalytic amount of DMAP.[47] Yields of the protected peptides were 85% as quantitated by spectrophotometric analyses of the fulvene–piperidine adduct at 300 nm. The protected heptapeptide resin was treated for 3 h at 20 °C with 5 mL of 95% TFA containing 0.5 mL of thioanisole, filtered, and then washed with TFA. After evaporation of the combined filtrate and washings, the free peptide was precipitated with diethyl ether. The solid material was dissolved in methanol/HOAc/H_2O (1.2:0.2:8) and passed through Sephadex LH 20; the major peaks were pooled and lyophilized. The powder was dissolved in 2 mL of 20% acetonitrile in 0.1% TFA and subjected to preparative HPLC (supra vide) using a 30-min gradient from 20 to 80% acetonitrile in 0.1% TFA. Two peaks were isolated as white powders with yields of 50 mg for **2** and 10 mg for **11**. Amino acid analyses gave the following molar ratios. **2**: Tyr, 0.94; Met, 1.96; Phe, 1.0; His, 0.96; Leu, 1.02; Asp, 0.94. **11**: Tyr, 0.95; Met, 2.1; Phe, 0.95; Leu, 1.0; Asp, 0.98.

[D-Phe^3]- and [D-Phe^3,des-His^4]deltorphin A (5 and 13, Respectively). The peptides were prepared using a modification of the above-described procedure for the synthesis of peptide **2** in which N^{α}-Fmoc-D-Phe and N^{α}-Boc-Tyr were substituted for N^{α}-Fmoc-Phe and N^{α}-Boc-D-Tyr. The protected peptide was removed from the resin with TFA–anisole and purified according to the procedure for peptide **2**; 65 mg of peptide **5** and 18 mg of peptide **13** were recovered. Amino acid analyses resulted in the following molar ratios. **5**: Tyr, 0.89; Met, 2.01; Phe, 0.97; His, 0.94; Leu, 1.0; Asp 0.95. **13**: Tyr, 0.85; Met, 1.97; Phe, 1.01; Leu, 1.0; Asp, 0.95.

[D-His^4]deltorphin A (6). As in the solid-phase synthesis for peptide **2**, N^{α}-Boc-Tyr and N^{α}-Fmoc-D-His(Trt) were substituted for N^{α}-Boc-D-Tyr and N^{α}-Fmoc-His(Bum). N^{α}-Fmoc-D-His-(Trt)-OH (0.8 mmol) was introduced by coupling with DIC (0.8 mmol) in the presence of a catalytic quantity of DMAP.[43] The protected peptide was removed from the resin with TFA–thioanisole; 67 mg of white powder was recovered. Amino acid analyses gave the following composition: Tyr, 0.98; Met, 1.92; Phe, 1.02; His, 0.89; Leu, 1.0; Asp, 0.99.

[D-Leu^5]- and [D-Leu^5,des-His^4]deltorphin A (7 and 14, Respectively). The analogues were prepared by substituting N^{α}-Fmoc-D-Leu and N^{α}-Boc-Tyr instead of N^{α}-Fmoc-Leu and N^{α}-Boc-D-Tyr using the method for the synthesis of peptide **2**. The yields of purified peptides were 47 mg of peptide **7** and 14 mg of peptide **14**. Results of amino acid analyses were as follows. **7**: Tyr, 0.95; Met, 1.94; Phe, 0.98; His, 0.96; Leu, 1.0; Asp, 0.97. **14**: Tyr, 0.95; Met, 1.96; Phe, 0.99; Leu, 0.98; Asp, 0.97.

[D-Met^6]- and [Des-His^4,D-Met^6]deltorphin A (8 and 15, Respectively). The peptides were prepared by substituting N^{α}-Fmoc-D-Met and N^{α}-Boc-Tyr in lieu of N^{α}-Fmoc-Met and N^{α}-Boc-D-Tyr in the synthesis of compound **2**. Yields of purified peptides were 55 mg of peptide **8** and 12 mg of peptide **15**. Results of amino acid analyses were as follows. **8**: Tyr, 0.91; Met, 2.02; Phe, 0.99; His, 0.95; Leu, 1.0; Asp, 0.94. **15**: Tyr, 0.96; Met, 1.94; Phe, 0.97; Leu, 1.0; Asp, 0.89.

[D-Asp^7]- and [Des-His^4,D-Asp^7]deltorphin A (9 and 16, Respectively). N^{α}-Fmoc-D-Asp(OtBu) and N^{α}-Boc-Tyr were substituted for N^{α}-Fmoc-Asp(OtBu) and N^{α}-Boc-D-Tyr as de-

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scribed for peptide 2. The yields of purified peptides were 48 mg of peptide 9 and 10 mg of peptide 16. Amino acid compositions were as follows. 9: Tyr, 0.86; Met, 2.01; Phe, 0.98; His, 0.88; Leu, 1.0; Asp, 0.97. 16: Tyr, 0.95; Met, 1.89; Phe, 0.98; Leu, 1.0; Asp, 0.95.

[D-Ala²]- and [D-Ala²,des-His⁴]deltorphin A (3 and 12, Respectively). N^{α}-Fmoc-D-Ala was substituted for N^{α}-Fmoc-D-Met in the method for the synthesis of peptide 1.[7,13] The yields were 63 mg for peptide 3 and 11 mg for peptide 12. Amino acid compositions gave the following. 3: Tyr, 0.91; Ala, 1.01; Phe, 0.99; His, 0.94; Leu, 1.0; Met, 0.96; Asp, 0.95. 12: Tyr, 0.94; Ala, 0.97; Phe, 0.98; Leu, 1.0; Met, 0.91; Asp 1.02.

[D-Nle²]deltorphin A (4). N^{α}-Fmoc-D-Nle was substituted for N^{α}-Fmoc-D-Met during synthesis.[7,13] The deprotected peptide was purified by two additional chromatographic steps to yield 21 mg of product. Final amino acid composition was Tyr, 0.87; Nle, 1.02; Phe, 0.97; His, 0.89; Leu, 1.0; Met, 0.95; Asp, 0.97.

[D-Met²]- and Deltorphin B (18 and 17, Respectively). Deltorphin B was synthesized as described.[7] N^{α}-Fmoc-D-Met replaced N^{α}-Fmoc-D-Ala in the synthesis of [D-Met²]deltorphin B (18). The amino acid composition, normalized after a 72-h hydrolysis, was Tyr, 0.91; Met, 0.95; Phe, 0.99; Glu, 0.91; Gly, 1.0; Val, 1.67.

Receptor Assays. Rat brain synaptosomes were obtained from Sprague-Dawley male rats, based on the method of Chang and Cuatrecasas,[48] as previously described.[6-8,49,50] The binding assays for δ receptors utilized 0.62 pmol [³H]DPDPE (DuPont/NEN) in a reaction mixture containing 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1 μM bestatin, 4 μg of bacitracin, 8% glycerol, 32 μg/mL soybean trypsin inhibitor, and 100 μM (phenylmethyl)sulfonyl fluoride; μ receptor assays used 1.28 nM [³H]DAGO (Amersham) with 1 M $MgCl_2$ in addition to the above buffer and protease inhibitors. After a 2-h incubation at 22 °C, the duplicate samples were rapidly filtered through wetted Whatman GF/C glass microfilters and washed with 3 × 2 mL 50 mM Tris-HCl, pH 7.5, containing 1 mg/mL BSA; the filters were dried at 75 °C and counted in a β counter using CytoScint (ICN) fluorophore. Peptides were assayed at 4–9 concentrations covering 2–3 orders of magnitude with n = 3–8; 3–5 synaptosomal preparations were used to ensure the statistical reliability of SEM. The equation of Cheng and Prusoff[51] was employed to calculate K_i values.

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Synthesis and Biological Activity of Novel Folic Acid Analogues: Pteroyl-*S*-alkylhomocysteine Sulfoximines

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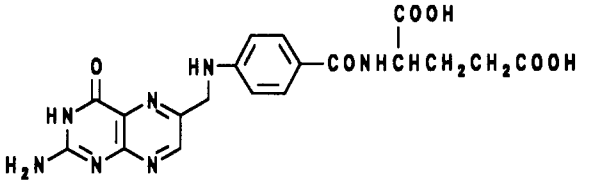
The synthesis of a novel series of γ-substituted folic acid analogues, pteroyl-*S*-alkyl-DL-homocysteine (*RS*)-sulfoximines, and the corresponding *S*-methylhomocysteine sulfone is described. Side reactions of the sulfoximine groups of the amino acid ester reactants were considered. The correct structures of the isolated target compounds were confirmed by NMR and FAB/MS excluding other alternatives. The replacement of the γ-COOH of the glutamate moiety of folic acid with *S*-alkylsulfoximine groups or *S*-methylsulfone did not affect the substrate activity of the vitamin for dihydrofolate reductase. The resulting tetrahydrofolate analogues could serve as cofactors for the thymidylate synthase cycle of murine leukemia L1210 cells in situ. The analogues inhibited the growth of these cells in culture with 2 orders of magnitude lower IC_{50} values [(2–4) × 10^{-4} M] than the parent folic acid.

Folic acid coenzymes play an important role in amino acid metabolism and are essential for the biosynthesis of nucleic acids.[1] They exist in the cell as tetrahydrofolyl poly-γ-glutamate conjugates, which are the preferred substrates (cofactors) for most folate-requiring enzymes.[2,3] Conversion to polyglutamates, which is catalyzed by folylpolyglutamate synthetase (FPGS), contributes significantly to the intracellular retention of tetrahydrofolate cofactors.[2] A variety of structural analogues of folic acid, exemplified by methotrexate, is capable of undergoing intracellular polyglutamylation, which may play an important role in the antitumor activity of these antifolates.[4] Since FPGS is essential for cellular viability,[5,6] it represents a potential target for chemotherapy.[7,8]



Folic acid (PteGlu)

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