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Para-Substituted Phe[3] Deltorphin Analogues: Enhanced Selectivity of Halogenated Derivatives for δ Opioid Receptor Sites

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The δ-selective opioid peptide deltorphin C(H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2) (DEL C) was modified by para-substitution of Phe[3] with halogens (F, Cl, Br, I), amino, or nitro groups. The bioactive potencies in peripheral tissues and brain receptor selectivities of these analogues depended upon the particular substituent; peptides containing halogen substituents exhibited the least disruptive effect. In the mouse vas deferens (MVD) bioassay, [*p*-ClPhe[3]]DEL C displayed equivalent bioactivities to DEL C; in combination with the guinea pig ileum (GPI) bioassay, [*p*-ClPhe[3]]DEL C and [*p*-BrPhe[3]]DEL C exhibited marked preference for δ sites (IC_{50}GPI/IC_{50}MVD = 11 250 and 6 363, respectively), which are ~4- and 2-fold greater than DEL C. In a receptor binding assay, none of the halogenated analogues had δ affinities (K_i) exceeding that of DEL C; however, in terms of δ selectivity ($K_i\mu/K_i\delta$), [*p*-BrPhe[3]]DEL C was nearly twice as selective as DEL C, while [*p*-FPhe[3]]-DEL C was equivalent, and [*p*-IPhe[3]]DEL C only 25% less selective. The only correlation evident with the halogenated derivatives occurred between IC_{50}GPI and $K_i\mu$ (r = 0.814) rather than between δ receptor studies (MVD or $K_i\delta$); interestingly, IC_{50}GPI also correlated with K' (r = 0.982). The *p*-amino or *p*-nitro substituents of Phe[3] in DEL C and DEL B (= [Glu[4]]DEL C) were deleterious for bioactivity (MVD) (losses ranged from 400- to ~8 000-fold) and in receptor binding assays, where δ affinities decreased 140- to 840-fold and δ selectivities by 34- to 380-fold. *p*-Nitro-Phe[3] was the most detrimental substitution for all the parameters measured for both deltorphins: the loss in MVD activity, however, was less with DEL B than with DEL C, which was the opposite for δ receptor affinity.

Introduction[1]

Halogenated derivatives (F, Cl, Br, and I) of Phe[4] in the conformationally restricted, cyclic [D-Pen[2,5]]enkephalin[2,3] and metkephamid[4] enhanced δ affinity to brain membrane receptors and bioactive δ selectivity in classical pharmacological assays designed for measuring δ and μ opioid receptors.[2] Similarly, halogenation of Phe[3] in the conformationally constrained (cyclic) tetrapeptide analogues of JOM-13 also led to higher δ affinities.[5] Interestingly, these observations led to the development of methods for the photoaffinity labeling of opioid receptors,[6] the isotopic

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(1) Symbols and abbreviations are in accord with the recommendations of the IUPAC-IUB Commission on Nomenclature (*J. Biol. Chem.* 1972, *247*, 977). Other abbreviations include: Boc, *tert*-butyloxycarbonyl; DAGO, [D-Ala[2],NMePhe[4],Gly-ol[5]]enkephalin; DCC, dicyclohexylcarbodiimide; DEL B, deltorphin B = [Glu[4]]DEL C, is known as [D-Ala[2]]-deltorphin II;[15] DEL C, deltorphin C (H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2) is also called [D-Ala[2]]deltorphin I;[15] DMF *N*,*N*-dimethylformamide; DPDPE, cyclic[D-Pen[2,5]]enkephalin; Et_2O, diethyl ether; FAB-MS, fast atom bombardment mass spectrometry; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; JOM-13, H-Tyr-D-Cys-Phe-D-Pen-OH; MVD, mouse vas deferens; NMM, *N*-methylmorpholine; NMR, nuclear magnetic resonance; O*t*Bu, *tert*-butyl ester; OSu, *N*-succinimidoyl; PITC, phenyl isothiocyanate; TFA, trifluoroacetic acid.

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labeling of the cyclic opioid peptides with ^{125}I at the para position of the phenylalanine ring,[7] and the application, along with [^{3}H][*p*-ClPhe^4]DPDPE,[3] in the pharmacokinetics of tissue distribution after intravenous administration of radiolabeled peptides;[8] para iodination was also used as an intermediary step in the tritiation of DEL B.[9] Other modifications on the electronic nature of the benzyl ring of phenylalanine in opioid peptides, such as the *p*-nitro derivatives of Phe^4 in cyclic[10,11] or linear[12,13] enkephalin derivatives and Phe^3 in JOM-13,[5] produced agonists with an enhancement in δ selectivity apparently due to the "electrophilic aromatic character" of the modified phenylalanine.[10] On the other hand, β-methyl-*p*-NO_2Phe^4 derivatives of conformationally restricted enkephalin analogues yielded nonselective peptides.[14]

Data over the past several years revealed that amphibian-derived deltorphins exhibit some of the highest biological potencies of opioid peptides in pharmacological bioassays[15,16] and receptor selectivities with rat brain preparations.[15,17-19] Since the deltorphins exhibit exceptional discriminatory properties between δ and μ receptor sites in those assay systems, these naturally occurring peptides seemed to be the logical source to launch structure–activity studies for the requirements on opioid peptide–receptor interactions. Analyses of deltorphin analogues were conducted to determine receptor selectivity and biological responsiveness at μ and δ binding sites in order to elucidate the molecular and potential topographical requirements of opioid ligands; they are essentially inactive with respect to κ receptor sites.[15] Strategies in the search for additional highly selective deltorphin ligands involved investigations on the systematic replacement of amino acids,[20-24] derivatization of functional groups,[24] shifted sequences,[24] and C-terminally abbreviated peptides.[20-22,24,26] Recently, we examined the effect of changing the stereospecificity,[18] altering the electronic and lipophilic nature of amino acid side chains,[20,23,27] and including various hydrophobic-conferring substituents in deltorphins.[20,27] These data taken together reveal that the deltorphins require a precise spatial orientation of the side chains[18] and a specific amino acid sequence.[20,24,25,27]

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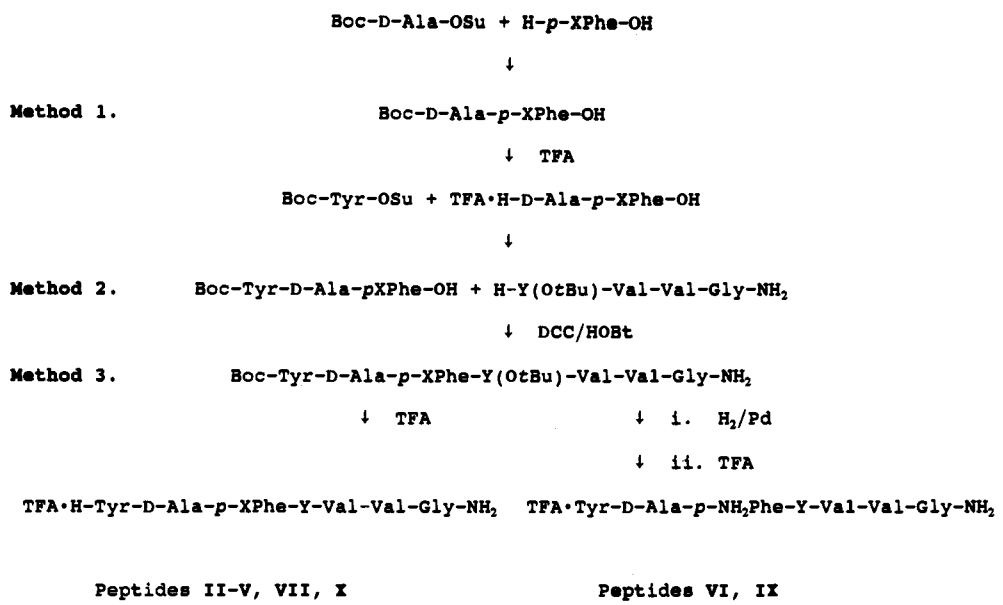

Figure 1. Scheme for the synthesis of para-substituted Phe³ deltorphin analogues. X = para-substituted F, Cl, Br, I, NO_2, or NH_2 groups and Y = Asp or Glu.

Rationale

One essential structural difference between the enkephalins and the deltorphin family of peptides resides in the N-terminal sequence. Conformational analyses of DEL C,[28] as well as dermorphin,[29] provided evidence for the existence of a type II′ β-turn in solution, in which the critical residues are Tyr¹, D-Ala², and Phe³.[18,28,29] On the basis of NMR data, the D-conformation of the Ala² side chain apparently stabilizes the β-turn through intramolecular interaction between the methyl hydrogens and Phe³.[28,29] Other investigations examined the effect of directly modifying the amino group of Tyr¹ in DEL C (Salvadori et al., unpublished results) and the spatial orientation of its aromatic side chain on receptor binding activity in deltorphin A[18] or dermenkephalin;[16,19,24] the proper spatial alignment of the benzyl side chain Phe³ is likewise required.[18] Therefore, in this study we turned our attention to the pivotal nature of Phe³ in deltorphins[18,25,27,28] and sought to probe the effect of substitutions at the para position of Phe³. We evaluated the activity of these DEL C analogues by pharmacological bioassays and radioligand binding assays for μ and δ receptors. However, due to the enhanced δ selectivity and bioactive potency of DEL B,[15,17] we further sought to determine if the single substitution of Glu⁴ for Asp⁴ in DEL B containing *p*-amino or *p*-nitro groups in Phe³ might overcome the deleterious effect on bioactivity observed with the inclusion of *p*-nitro-Phe³ in DEL C.[22] Moreover, if we could correlate the biological activity, receptor assays, or chemical properties of the para-modified Phe³ in a defined series, such as with the halogenated analogues, then we might be able to reach conclusions and interpretations which might be relevant to the future design of opioid agonists or antagonists based on dermorphin–deltorphin structural principles.[5]

General Methods Involved in Peptide Synthesis

The *p*-Phe³ derivatized heptapeptides listed in Table III were synthesized by the fragment 3 + 4 condensation technique[25] as illustrated in Figure 1. Active esters and DCC/HOBt were employed in the coupling reactions. Protection of amino acids used Boc for α-amino functions and *tert*-butyl groups for the aspartic acid side chain; tyrosine was unprotected. Boc and O*t*Bu protecting groups were removed by treating the peptide with aqueous 90% TFA for 0.5–1 h; TFA was removed under reduced pressure and the residue triturated with Et_2O.[25] Purification was achieved by crystallization from MeOH/Et_2O and, in some cases, by open column chromatography (270 × 7 cm per 0.7–1 g material) utilizing silica gel 60 (70–230 mesh; Merck) and a gradient from 10% to 60% MeOH in $CHCl_3$.[18,20,25] Crude material after deprotection of peptides I–X was dissolved in 10% aqueous AcOH and passed through Sephadex G25 (2 × 50 cm) equilibrated and eluted in AcOEt/pyridine/AcOH/H_2O (6:2.2:0.6:1.1, v/v/v/v). The major absorbance peak was collected and lyophilized. The powders (~90 mg) were dissolved in 1 mL of an equal mixture of mobile phases A and B and then purified by HPLC. The main absorbance peak was lyophilized as a white powder, and the chemical characteristics of the purified peptides are given in Table I.[18,20] The homogeneity of the purified products were assessed by TLC, HPLC, amino acid analysis, and FAB-MS, and further characterized by NMR, melting point, and optical rotation (Table I).

Results and Discussion

The spectrum of bioactivity responses and the receptor binding analyses of the deltorphins and their analogues are presented in Tables II and III, respectively. DEL C (I) and DEL B (VIII) exhibit high δ affinities and exceptionally high δ selectivities, comparable to previous estimates using assays with rat brain synaptosomes[15,20,23]

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

no.	peptide	TLC R_f values I	II	III	HPLC T_R, min	K'	mp, °C	$[\alpha]^{20}_D$, deg	M^+ calcd	obsd
I	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	0.45	0.38	0.12	8.0	2.98	162–166	−9.2	769	769
II	Tyr-D-Ala-*p*-FPhe-Asp-Val-Val-Gly-NH_2	0.65	0.63	0.23	9.8	3.66	167–169	−3.8	787	787
III	Tyr-D-Ala-*p*-ClPhe-Asp-Val-Val-Gly-NH_2	0.68	0.65	0.24	10.1	3.81	158–162	−2.7	803	803
IV	Tyr-D-Ala-*p*-BrPhe-Asp-Val-Val-Gly-NH_2	0.69	0.69	0.20	11.0	4.24	170–174	−4.0	848	847, 849
V	Tyr-D-Ala-*p*-IPhe-Asp-Val-Val-Gly-NH_2	0.72	0.70	0.18	11.7	4.57	177–180	−3.2	895	895
VI	Tyr-D-Ala-*p*-NH_2Phe-Asp-Val-Val-Gly-NH_2	0.50	0.37	0.26	8.15	4.05	202–204	−6.3	784	784
VII	Tyr-D-Ala-*p*-NO_2Phe-Asp-Val-Val-Gly-NH_2	0.53	0.47	0.34	11.1	6.88	210–213	−2.1	814	814
VIII	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2	0.62	0.45	0.31	10.9	6.71	185–187	−14.7	783	783
IX	Tyr-D-Ala-*p*-NH_2Phe-Glu-Val-Val-Gly-NH_2	0.48	0.50	0.69	7.9	4.59	193–197	−6.43	798	798
X	Tyr-D-Ala-*p*-NO_2Phe-Glu-Val-Val-Gly-NH_2	0.56	0.51	0.87	12.3	6.45	205–207	−1.1	828	828

[a] The TLC solvent systems, HPLC conditions, and optical rotation determinations are given in Experimental Section.

Table II. Bioactivity Profiles of Para-Substituted Phe[3] Deltorphin Analogues[a]

no.	peptide	IC_{50} MVD	GPI	IC_{50}GPI/IC_{50}MVD
I	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	0.14 ± 0.06	420 ± 95	3 000
II	Tyr-D-Ala-*p*-FPhe-Asp-Val-Val-Gly-NH_2	3.5 ± 0.4	1 100 ± 150	314
III	Tyr-D-Ala-*p*-ClPhe-Asp-Val-Val-Gly-NH_2	0.16 ± 0.02	1 800 ± 200	11 250
IV	Tyr-D-Ala-*p*-BrPhe-Asp-Val-Val-Gly-NH_2	1.1 ± 0.15	7 000 ± 800	6 363
V	Tyr-D-Ala-*p*-IPhe-Asp-Val-Val-Gly-NH_2	17 ± 2	>10 000	>590
VI	Tyr-D-Ala-*p*-NH_2Phe-Asp-Val-Val-Gly-NH_2	1 100 ± 250	>10 000	>9
VII	Tyr-D-Ala-*p*-NO_2Phe-Asp-Val-Val-Gly-NH_2	1 200 ± 270	>10 000	>8
VIII	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2	0.75 ± 0.10	5 200 ± 1 430	6 933
IX	Tyr-D-Ala-*p*-NH_2Phe-Glu-Val-Val-Gly-NH_2	300 ± 67	>10 000	>33
X	Tyr-D-Ala-*p*-NO_2Phe-Glu-Val-Val-Gly-NH_2	430 ± 94	>10 000	>23

[a] The assay conditions are detailed in Experimental Section. The IC_{50} values are mean, nM ± SEM with $n = 5$.

Table III. Receptor Analyses of Para-Substituted Deltorphins[a]

no.	peptide	$K_i\delta$	$K_i\mu$	$K_i\mu/K_i\delta$
I	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	0.24 ± 0.06 (6)	272 ± 50 (11)	1135
II	Tyr-D-Ala-*p*-FPhe-Asp-Val-Val-Gly-NH_2	0.51 ± 0.07 (4)	554 ± 65 (4)	1086
III	Tyr-D-Ala-*p*-ClPhe-Asp-Val-Val-Gly-NH_2	1.58 ± 0.11 (4)	287 ± 50 (4)	181
IV	Tyr-D-Ala-*p*-BrPhe-Asp-Val-Val-Gly-NH_2	2.39 ± 0.39 (4)	4787 ± 417 (4)	2003
V	Tyr-D-Ala-*p*-IPhe-Asp-Val-Val-Gly-NH_2	1.62 ± 0.33 (5)	1413 ± 88 (3)	872
VI	Tyr-D-Ala-*p*-NH_2Phe-Asp-Val-Val-Gly-NH_2	33.9 ± 5.10 (3)	506 ± 50 (3)	15
VII	Tyr-D-Ala-*p*-NO_2Phe-Asp-Val-Val-Gly-NH_2	71.8 ± 13.8 (4)	2391 ± 401 (3)	33
VIII	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2	0.12 ± 0.03 (6)	638 ± 55 (5)	5317
IX	Tyr-D-Ala-*p*-NH_2Phe-Glu-Val-Val-Gly-NH_2	48.1 ± 6.8 (3)	2460 ± 330 (3)	51
X	Tyr-D-Ala-*p*-NO_2Phe-Glu-Val-Val-Gly-NH_2	100.6 ± 23.7 (3)	1429 ± 175 (3)	14

[a] The inhibition constant K_i (mean, nM ± SEM, with n repetitions in parenthesis) was calculated according to Cheng and Prusoff:[37] $K_i = (IC_{50}/(1 + L/K_d))$, 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.

and biological potencies determined by their spectra of activities in isolated tissue assays.[15,16]

The halogenated DEL C analogues involved a progressive decrease, in the series F < Cl < Br < I, in μ bioactivities (IC_{50}GPI) relative to the parent peptide (I); μ bioactivities apparently decreased with increased bulkiness of the substituted halogen and inverse to their electronegativity (I = 2.4, Br = 2.8, Cl = 3.0, F = 4.0). In terms of δ bioactivity, however, *p*-ClPhe[3] had comparable activity to DEL C and the highest δ biological potency (IC_{50}GPI/IC_{50}MVD) (Table II). In general, the δ bioactivity (MVD) of the halogenated peptides varied independently with any apparent properties of the substituent. This observation differs from that of the enkephalin analogues: the most δ-selective analogue was cyclic [*p*-IPhe[4]]DPDPE,[2] while the linear [*p*-FPhe[4]]metkephamid had high receptor binding and biological activity.[4] The orientation of Phe[4] in the conformationally restrained enkephalin-derived peptides[2] and metkephamid[4] in comparison to that of Phe[3] in the deltorphins could indicate significant differences in the alignment of the aromatic ring of Phe[3] in the receptor site.[28]

In the receptor binding characteristics of the halogenated peptides, δ selectivity ($K_i\mu/K_i\delta$) of the *p*-BrPhe[3] derivative (IV) was nearly twice (= 2 003) that of DEL C (I) (= 1 135) due to a nearly 20-fold loss of μ binding. On the basis of concomitant 2-fold loss in both δ and μ receptor binding, *p*-FPhe[3] (II) was essentially equivalent to the δ selectivity of DEL C (I). The δ selectivity of *p*-IPhe[3] (V) was marginally reduced due to 8- and 5-fold losses in δ and μ receptor affinities, respectively; however, the *p*-chloro derivative (III) exhibited an 8-fold decrease in selectivity solely due to a comparable loss in δ affinity. In contrast, the *p*-chloro derivatives of the conformationally constrained halogenated analogues *p*-ClPhe[4] (DPDPE)[2] or *p*-ClPhe[3] (JOM-13)[5] had the largest increase in δ affinity and selectivity.

Although a correlation exists between δ binding and MVD bioactivity with halogenated DPDPE analogues,[2] the only relationship observed between receptor affinity and biological activity of the halogenated DEL C analogues was found between IC_{50}GPI and $K_i\mu$ ($r = 0.814$) (extending approximately 1.5 orders of magnitude in both assay systems) (Figure 2); no correlation was seen between measurements for δ parameters. This is indeed a singularly uncommon observation since DEL C is a highly δ selective agonist.[15,25] These results and the interesting correlation between GPI bioassays and K' (Figure 3) is open to

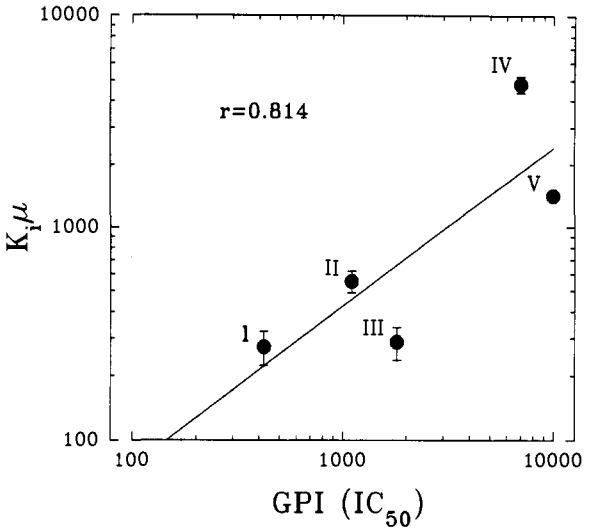

Figure 2. Correlation between bioactive potency in the peripheral guinea pig ileum (GPI) assay (IC_{50}) and μ receptor binding assay ($K_i\mu$) using rat brain synaptosomes. The correlation coefficient, $r = 0.814$. Error bars lie within the dimensions of the figure symbol when absent.

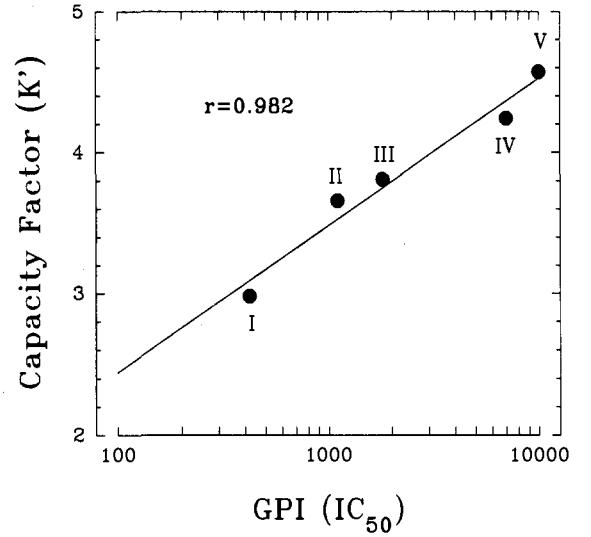

Figure 3. Correlation between the bioactivity observed with the guinea pig ileum (GPI) assay (IC_{50}) and the capacity factor (K') determined by analytical HPLC (Table I). The correlation coefficient, $r = 0.982$.

interpretation, but not without a degree of caution as suggested by Toth et al.[2] due to inherent differences between central and peripheral receptors. (a) Central and peripheral μ receptors may be more similar than the comparable δ receptors due a correlation observed between brain μ receptors and peripheral activity (suppression of gastric acid secretion) with high μ affinity analogues of dimeric dermorphin-related peptides.[30] (b) Another plausible explanation is that only one μ receptor subtype interacts with our deltorphin analogues, regardless of the tissue of origin, since these peptides are more selective for δ receptor sites.[15,17,19] (c) δ Receptor subtypes might explain the differential interaction exhibited by *p*-chloro-Phe³ (III) and *p*-bromo-Phe³-containing (IV) analogues in pharmacological bioassays of peripheral tissue (Table II) and receptor binding assays in brain membranes (Table III).

The para-substituted amino and nitro groups of Phe³ in either DEL C analogues (VI and VII) and DEL B analogues (IX and X) were detrimental for all bioassays (MVD and GPI) and δ receptor affinities. The MVD responses precipitously dropped by ~8 000-fold for both the *p*-amino (VI) and *p*-nitro derivatives (VII) of DEL C; the decreases for the corresponding analogues of DEL B (IX and X) were considerably less, only 400- to ~500-fold (Table II). Thus, the Glu⁴ residue, which differs from Asp⁴ in length of the side chain by a CH_2 group, modified the response of these DEL B analogues in the MVD bioassay. δ Receptor binding fell ~100- to 800-fold relative to both parent peptides; in this assay system, however, the differences were not substantial between the analogues of DEL B and DEL C (Table III). Interestingly, the *p*-nitro substituents of DEL C and DEL B analogues were consistently twice as deleterious as the *p*-amino derivatives on δ receptor affinity. A similar loss of receptor affinities and bioassay potencies was also reported by Schiller et al. for a *p*-nitro derivative of Phe³ in dermorphin and morphiceptin,[10] as well as with DEL C;[22] earlier we also concluded that the introduction of polar group in the aromatic ring of Phe³ of a dermorphin analogue decreased bioactivity.[31] A partial explanation for the detrimental effects of a *p*-nitro substituent on biological activity and receptor binding may involve the high electron-attracting dipole moment of NO_2 (3.97), which is considerably greater than those of the halogens (I = 1.30, Cl = 1.53, Br = 1.56) due to the positive charge carried by the nitrogen. However, this cannot explain the difference between the inhibitory properties exerted by the *p*-nitro and *p*-amino group, since $-NH_2$ is an electron donor with a dipole moment of 1.52.

The μ receptor binding of these *p*-amino and *p*-nitro analogues (VI, VII, IX, and X) also declined, but less than that observed for δ binding; δ selectivity of [*p*-NH_2Phe³]- (VI) and [*p*-NO_2Phe³]DEL C (VII) fell 75- and 34-fold, respectively, relative to that of DEL C (I), while the equivalent substitutions in DEL B (IX and X) led to a drop in δ selectivity of ~100- to 400-fold, respectively, relative to that of the parent compound (VIII). The loss in selectivity was greater with DEL B than DEL C since the former peptide is the most δ-selective natural opioid peptide identified to date.[15,23] This contrasts sharply with the increase in δ and μ affinities of the comparable substitution of Phe⁴ in enkephalin analogues.[10-13] Further, the incorporation of an azido group in an enkephalin, [*p*-N_3-Phe⁴,Met⁵]enkephalin,[6] also enhanced δ binding affinity; this indicated that para substituents on Phe⁴ augment the electronic properties of the benzyl ring to an extent that enables enkephalins to be more selectively recognized by the δ receptor. Based on the losses in the bioassay potencies of [*p*-NO_2Phe³]dermorphin[11] and [*p*-NO_2Phe³]-DEL C,[22] as well as the bioactivities (Table II) and receptor binding efficacies of our DEL C analogues (Table III), it appears that the *p*-nitro group on the benzyl ring of Phe³ [which also changes its hydrophobicity and pK_a (= 3.42)[10]] might modify the electronic milieu of the aromatic ring of

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Phe[3] which could effect the solution conformation of the peptide or prevent the appropriate alignment of the peptide in a receptor pocket or both. The positioning or orientation of the side chain of Phe[3] in linear deltorphins may be more stringent for receptor recognition than that which occurs with Phe[4] in the conformationally constrained[14] and linear enkephalins,[11] or Phe[3] in JOM-13 cyclic analogues.[5]

Conclusions

The electronegativity of the halogens and their increased molecular volumes,[2,4] carbon–halogen bond lengths (F = 1.40, Cl = 1.79, Br = 1.97, I = 2.16 Å), dipole moments, and the elevated lipophilicities of para-halogenated DEL C derivatives (Table I) influence the nature of the aromatic ring of Phe[3] and peptide conformation; however, individual differences exerted by or attributed to these parameters remain difficult to assess. Nonetheless, the potential involvement of the overall change in the lipophilicity of the halogenated DEL C peptides, rather than electronegativity per se,[2] was observed by the high degree of correlation (r = 0.982) which occurred between the μ bioactivity and the increase in K' (Table I and Figure 3); no apparent trend or correlation appeared between MVD or δ receptor binding and any chemical property attributable to the halogens. The dramatic decrease in δ receptor selectivity by p-amino and p-nitro groups, which primarily affected binding to the δ sites, was more pronounced than the changes seen with the para-halogenated peptides.

Thus, the selective changes in the electronic composition of the aromatic ring of Phe[3] of DEL C through the introduction of halogens at the para position differs sharply from those modifications brought upon by addition of p-amino or p-nitro groups. Although we have not investigated the effects of substitutions at the ortho or meta positions of Phe[3] in the deltorphins, it would appear safe to postulate that μ and δ receptor sites are exquisitely sensitive to changes in the chemical nature of the benzyl ring of a para-substituted Phe[3]. The data further suggest that these substituents contribute sufficient changes in electronic parameters[31] to modify binding to a δ receptor or subtype distinct from that of the enkephalin-derived peptides and other cyclic peptides.

Experimental Section

Analytical Methods. Melting points were determined on a Kofler apparatus and are uncorrected. Optical rotations were conducted in a Perkin-Elmer 241 polarimeter with a 10-cm water-jacketed cell using MeOH for the di- and tripeptides and DMF for the heptapeptides. TLC was performed on precoated plates of silica gel F_{254} (Merck, Darmstadt, Germany) using the following solvent systems: (I) 1-butanol/AcOH/H_2O (6:1:5, v/v/v); (II) AcOEt/pyridine/AcOH/H_2O (6:2:0.6:1.1, v/v/v/v); and (III) CH_2Cl_2/MeOH/benzene (8:2:1). The detecting solutions included ninhydrin, fluorescamine, or chlorine reagent. Amino acid analyses were carried out using PITC methodology (Pico-Tag, Waters-Millipore, Waltham, MA). Peptides (50–1 000 pmol) were hydrolyzed in sealed borosilicate tubes (4 × 50 mm) in 200 μL of 6 N HCl containing 1% phenol for 1 h at 150 °C; a Pico-Tag column (3.9 × 15 mm) separated the PITC amino acid derivatives. Elemental analyses were conducted after the products were dried for 12 h at 50 °C and 0.2 Torr.

Preparative HPLC used Waters Delta Prep 3000 with a Delta Pak C_{18} column (30 × 300 mm, 15 μm, 300 Å); mobile phases consisted of A, 10% acetonitrile–0.1% TFA (v/v), and B, 60% acetonitrile–0.1% TFA. Elution was conducted in a linear gradient to 80% B in 25 min at a flow rate of 30 mL/min with detection at 220 nm. Analytical HPLC was conducted with a Bruker LC 21-C liquid chromatography system fitted with a Bruker LC 313 UV variable wavelength detector; recording and quantification was accomplished through an Epson QX-10 computer system. The retention time (t_R) and capacity factor (K') were determined as follows: peptides I–V on Spherisorb C_{18} columns (250 × 4.6 mm, 5-μm diameter) and eluted by means of a linear gradient in the above mobile phases run to 80% B in 15 min at a flow rate of 2 mL/min; peptides VI–X on Vydac C18 columns (250 × 4.6 mm, 5-μm diameter) in a linear gradient to 60% solvent B in 25 min at a flow rate of 2 mL/min.

Specific Coupling Procedures. Method A. To a stirred solution of halogenated amino acid or halogenated dipeptide (1 mmol) in DMF (~10 mL/mmol) was added 1.1 equiv of NMM (2.2 equiv of NMM if the amino component is protonated); the mixture was cooled to 0 °C, treated with activated Boc-amino acid (OSu) (0.9 mmol), and the mixture was allowed to react at room temperature for 4 h. The solution was diluted with AcOEt (50 mL) and washed with brine, 0.5 N $KHSO_4$, and brine. The organic phase was dried ($MgSO_4$), filtered, and evaporated to dryness. The residue was crystallized from appropriated solvents or purified by column chromatography.

1. Boc-D-Ala-*p*-X-Phe-OH. Boc-D-Ala-OSu (10 mmol) was reacted with H-p-X-Phe-OH (11 mmol), and the crude dipeptide was crystallized from MeOH/Et_2O. Analytical data is given for the following halogenated dipeptides. X = F: 89% yield; mp 76–78 °C; $[\alpha]^{20}_D$ +41.1°; TLC R_f (I) 0.73, (II) 0.82, (III) 0.42. Anal. $C_{17}H_{23}N_2O_5F$. X = Cl: 85% yield; mp 69–72 °C; $[\alpha]^{20}_D$ +40.4°; TLC R_f (I) 0.77, (II) 0.84, (III) 0.47. Anal. $C_{17}H_{23}N_2O_5Cl$. X = Br: 78% yield; mp 94–96 °C; $[\alpha]^{20}_D$ +30.7°; TLC R_f (I) 0.79, (II) 0.88, (III) 0.48. Anal. $C_{17}H_{23}N_2O_5Br$. X = I: 89% yield; mp 90–92 °C; $[\alpha]^{20}_D$ +33.2°; TLC R_f (I) 0.81, (II) 0.91, (III) 0.51. Anal. $C_{17}H_{23}N_2O_5I$. X = NO_2: 80% yield; mp 74–77 °C; $[\alpha]^{20}_D$ −10.22°; TLC R_f (I) 0.92, (II) 0.78, (III) 0.37. Anal. $C_{17}H_{23}N_3O_7$.

2. Boc-Tyr-D-Ala-*p*-X-Phe-OH. Boc-Tyr-OSu (4 mmol) was reacted with H-D-Ala-p-X-Phe-OH as the TFA salt (4.5 mmoL) synthesized as in step 1 (Figure 1). The crude tripeptides were recrystallized from Et_2O. Analysis of the halogenated tripeptides are as follows. X = F: 91% yield; mp 115–117 °C; $[\alpha]^{20}_D$ +26.1°; TLC R_f (I) 0.67, (II) 0.85, (III) 0.43. Anal. $C_{26}H_{32}N_3O_7F$. X = Cl: 87% yield; mp 73–76 °C; $[\alpha]^{20}_D$ + 16.1°; TLC R_f (I) 0.69, (II) 0.86, (III) 0.47. Anal. $C_{26}H_{32}N_3O_7Cl$. X = Br: 84% yield; mp 120–121 °C; $[\alpha]^{20}_D$ +27.5°; TLC R_f (I) 0.70, (II) 0.87, (III) 0.55. Anal. $C_{26}H_{32}N_3O_7Br$. X = I: 90% yield; mp 98–100 °C; $[\alpha]^{20}_D$ +18.2°; TLC R_f (I) 0.73, (II) 0.90, (III) 0.56. Anal. $C_{26}H_{32}N_3O_7I$. X = NO_2: 77% yield; mp 112–115 °C; $[\alpha]^{20}_D$ −3.2°; TLC R_f (I) 0.87, (II) 0.78, (III) 0.41. Anal. $C_{26}H_{32}N_4O_9$.

Method B. To a stirred solution (0.5 M) of Boc-protected tripeptide (1 mmol) in DMF, the following were added at 0 °C: DCC (1.1 mmol), HOBt (1.1 mmol), and a precooled solution of the amino tetrapeptide component (1.1 mmol) in DMF (0.5 M). The reaction mixture was stirred at room temperature overnight. DCC was filtered off and the solution diluted with AcOEt (100 mL) and worked up as described for method A.

3. Boc-Tyr-D-Ala-*p*-X-Phe-Y(O*t*Bu)-Val-Val-Gly-NH_2. Boc-Tyr-D-Ala-p-X-Phe-OH (1 mmol) was reacted with H-Y(OtBu)-Val-Val-Gly-NH_2[19] (1.1 mmol) (Y = Asp or Glu). Crude heptapeptides were purified on silica gel columns (2 × 60 cm) by elution in CH_2Cl_2/MeOH/benzene (17:1:2, v/v/v). The fractions containing the pure compound were evaporated to dryness, and the residue was crystallized from AcOEt/Et_2O. Analytical data for the halogenated compounds are as follows. X = F and Y = Asp: 62% yield; mp 221–223 °C; $[\alpha]^{20}_D$ −3.2°; TLC R_f (I) 0.78, (II) 0.90, (III) 0.33. Amino acid analysis: Tyr 0.91; Ala 0.99; p-FPhe 0.97; Asp 1.05; Val 1.87; Gly 1.0. X = Cl: 66% yield; mp 170–172 °C; $[\alpha]^{20}_D$ −3.0°; TLC R_f (I) 0.8, (II) 0.93, (III) 0.34. Amino acid analysis: Tyr 0.91; Ala 0.97; p-ClPhe 0.98; Asp 0.97; Val 1.85; Gly 1.0. X = Br: 58% yield; mp 202–205 °C; $[\alpha]^{20}_D$ −3.9°; TLC R_f (I) 0.81, (II) 0.93, (III) 0.38. Amino acid analysis: Tyr 0.87; Ala 1.07; p-BrPhe 1.05; Asp 0.99; Val 1.9; Gly 1.0. X = I: 60% yield; mp 212–215 °C; $[\alpha]^{20}_D$ −9.6°; TLC R_f (I) 0.84, (II) 0.94, (III) 0.41. Amino acid analysis: Tyr 0.93; Ala 1.02; p-IPhe 0.89; Asp 1.03; Val 1.77; Gly 1.0. X = NO_2 and Y = Asp: 65% yield; mp 191–194 °C; $[\alpha]^{20}_D$ −5.4°; TLC R_f (I) 0.91, (II) 0.8, (III) 0.54. Amino acid analysis: Tyr 0.95; Ala 0.98; Asp 1.01; Val 1.87; Gly 1.0. X = NO_2 and Y = Glu: 71% yield; mp 189–191

°C; $[\alpha]^{20}_D$ -5.0°; TLC R_f (I) 0.93, (II) 0.81, (III) 0.55. Amino acid analysis: Tyr 0.89; Ala 1.02; Glu 0.98; Val 1.9; Gly 1.0.

Reduction of [*p*-NO_2Phe³]- to [*p*-NH_2Phe³]DEL C Heptapeptides. This reduction was carried out by hydrogenolysis in AcOH/MeOH (1:3, v/v) at atmospheric pressure and room temperature in the presence of 10% palladium on charcoal (catalyst to peptide ratio, 1:5, w/w). The reaction mixture was filtered through a Celite bed and evaporated to dryness. The residue was treated without further characterization as described above in the deprotection procedures.

Bioassays. A 2–3-cm segment of guinea pig ileum (GPI) was mounted in a 20-mL organ bath using the method of Kosterlitz and Watt.[32] The tissue was bathed in Kreb's solution containing 70 μM hexamethonium bromide and 0.125 μM mepyramine maleate and aerated with 95% O_2/5% CO_2 at 36 °C. The ileum was stimulated transmurally with square-wave electrical pulses of 0.5 ms duration at a frequency of 0.1 Hz. Unless otherwise stated, the strength of the stimulus was 1.5 times that which produced a maximal twitch (~30 V). The contractions were recorded isotonically at a magnification ratio of 1:15. The concentration of peptide (nM) required to inhibit the amplification of the electrically induced twitch by 50% is given as the IC_{50} value. A single vas deferens (MVD) from a mature mouse (30–35 g) was dissected and suspended in 4 mL of modified Kreb's solution[33] aerated with 95% O_2/5% CO_2 at 33 °C. The twitch, induced by field stimulation (0.1 Hz for 1 ms at 40 V), was recorded by means of an isometric transducer.

Dose–response curves were constructed by addition of appropriate amounts of peptides in 10–100 μL of Kreb's solution to the aerated baths; three to four washings of the tissue were conducted at 10-min intervals between each dose. A log–dose curve was determined using dermorphin or morphine standards for each tissue preparation, and the IC_{50} values of the compounds were normalized according to published procedures.[34] The K_e values for the antagonists naloxone or *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu-OH (ICI 174,864) were in the range of 1–2 nM obtained from the ratio of the IC_{50} values obtained in the presence and absence of a fixed antagonist concentration;[19] e.g., in the GPI bioassay for compound V the K_e for naloxone was 1.68 ± 0.37 nM, and in the MVD bioassay for compound IX, the K_e for ICI 174,864 was 1.92 ± 0.75 nM.

Radioreceptor Assays. Synaptosomal preparations (P_2) were prepared from Sprague-Dawley CD male rats according to Chang and Cuatrecasas[35] as described.[17,18,30,36] The synaptosomes were preincubated to remove endogenous opioids and washed thrice by centrifugation before storage in buffer (50 mM HEPES, pH 7.5, containing 20% glycerol, 50 μg/mL soybean trypsin inhibitor, and 1 mM dithiothreitol) in 4-mL aliquots at -80 °C.[30] Under these conditions, synaptosomes could be thawed and refrozen without deleterious effects on binding.[30]

Receptor affinities were measured by filtration through Whatman glass fiber filters (GF/C) and washed 3 × 2 mL with ice-cold buffered BSA within 5 s.[18] Steady-state binding were conducted at 22 °C for 120 min in duplicate assays containing 1.6 mg of protein using 0.62 nM [³H]DPDPE (34.3 Ci/mmol, New England Nuclear-DuPont, Boston, MA) to label δ sites and 0.68 nM [³H]DAGO (60 Ci/mmol, Amersham, Arlington, IL) for μ sites as detailed previously.[30] The difference in radioligand bound to that displaced by either 1–2 μM DPDPE or DAGO, respectively, is defined as specific binding. Filters were dried at 80 °C, and bound radioactivity was measured by liquid scintillation spectrometry using CytoScint (ICN, Irvine, CA). The K_i values were calculated from the IC_{50} values according to Cheng and Prusoff.[37] Correlations between bioactivity profiles and receptor affinities were analyzed with SigmaPlot (v. 4.04) (Jandel Scientific).

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