Topographically Designed Analogues of [D-Pen,D-Pen^5]enkephalin[1]

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The conformationally restricted, cyclic disulfide-containing δ opioid receptor selective enkephalin analogue [D-Pen^2,D-Pen^5]enkephalin (1, DPDPE) was systematically modified topographically by addition of a methyl group at either the *pro-S* or *pro-R* position of the β carbon of an L-Phe^4 or D-Phe^4 residue to give [(2*S*,3*S*)-β-MePhe^4]DPDPE (2), [(2*R*,3*R*)-β-MePhe^4]DPDPE (3), [(2*S*,3*R*)-β-MePhe^4]DPDPE (4), and [(2*R*,3*S*)-β-MePhe^4]DPDPE (5). The four corresponding isomers were prepared in which the β-methylphenylalanine residue was *p*-nitro substituted, that is with a β-methyl-*p*-nitrophenylalanine (β-Me-*p*-NO_2Phe) residue, to give [(2*S*,3*S*)-β-Me-*p*-NO_2Phe^4]DPDPE (6), [(2*R*,3*R*)-β-Me-*p*-NO_2Phe^4]DPDPE (7), [(2*S*,3*R*)-β-Me-*p*-NO_2Phe^4]DPDPE (8), and [(2*R*,3*S*)-β-Me-*p*-NO_2Phe^4]DPDPE (9), respectively. The potency and selectivity (δ vs μ opioid receptor) were evaluated by radioreceptor binding assays in the rat brain using [^{3}H]CTOP (μ ligand) and [^{3}H]DPDPE (δ ligand) and by bioassay with mouse vas deferens (MVD, δ receptor assay) and guinea pig ileum (GPI, μ receptor assay). The eight analogues of DPDPE showed highly variable binding and bioassay activities particularly at the δ opioid receptor (4 orders of magnitude), but also at the μ opioid receptor, which led to large differences (3 orders of magnitude) in receptor selectivity. For example, [(2*S*,3*S*)-β-MePhe^4]DPDPE (2) is 1800-fold selective in binding to the δ vs μ receptor, making it one of the most selective δ opioid receptor ligands in the enkephalin series as assessed by the rat brain binding assay, whereas the corresponding (2*R*,3*R*)-β-Me-*p*-NO_2Phe-containing analogue 9 is only 4.5-fold selective (nonselective) in this same assay. On the other hand, in the bioassay systems, [(2*S*,3*S*)-β-Me-*p*-NO_2Phe^4]DPDPE (5) is more potent than DPDPE and 8800-fold selective for the MVD (δ receptor) vs the GPI (μ receptor), making it the most highly selective ligand in this series for the δ opioid receptor on the basis of these bioassays. In these assay systems, the (2*R*,3*S*)-β-MePhe^4-containing analogue 5 had very weak potency and virtually no receptor selectivity (4.4-fold). These results demonstrate that topographical modification alone in a conformationally restricted peptide ligand can significantly modulate both potency and receptor selectivity of peptide ligands that have multiple sites of biological activity and suggest that this approach may have general application to peptide ligand design.

Introduction

There is now overwhelming evidence from pharmacological considerations[2-4] and from structure–activity studies[5] that the endogenous opioid peptides methionine enkephalin, leucine enkephalin, and dynorphin interact with several subtypes of opioid receptors (μ, δ, κ and possibly others) that may mediate different responses. An important goal in this research has been to develop highly selective ligands for these opioid receptors to aid in elucidating the function(s) of opioid receptors and their subtypes and in determining the structural and conformational properties important to their biological activities.

Our approach in this area has involved the application of conformational constraints utilizing cyclization and other conformational restrictions.[6,7] In our laboratory, this approach has led to the development of ligands among the most highly selective for μ, δ, and κ receptors.[8-14] In the case of analogues for the δ opioid receptor, a highly selective lead compound has been the cyclic enkephalin analogue [D-Pen^2,D-Pen^5]enkephalin (H-Tyr-D-Pen-Gly-Phe-D-Pen-OH, DPDPE), which was shown to be well over 1000 times selective for the δ vs μ receptor by using the mouse vas deferens and guinea pig ileum bioassays. This and other assays indicate that until recently DPDPE has been among the most selective δ receptor ligands known.[5,8,15,16] We have sought to further improve the potency and selectivity of DPDPE by consideration of the conformational properties[17] of this constrained cyclic peptide. Of particular interest from this perspective were the conformation properties of the aromatic side chain groups of the Tyr^1 and Phe^4 residues. We have examined the structural and stereochemical properties of the Phe^4 aromatic ring that are most compatible with interaction with the δ opioid receptor and for selection of the δ vs μ opioid receptor (DPDPE has essentially no interaction with the κ opioid receptor),[8] primarily by substitution on the

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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). All optically active amino acids are of the L variety unless otherwise noted. Other abbreviations used are as follows: β-MePhe, β-methylphenylalanine; Pen, penicillamine; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; [D-Phe^4]DPDPE, Tyr-D-Pen-Gly-D-Phe-D-Pen; [*p*-ClPhe^4]-DPDPE, Tyr-D-Pen-Gly-*p*-Cl-Phe-D-Pen; GPI, guinea pig ileum; MVD, mouse vas deferens; FAB-MS, fast atom bombardment mass spectrometry; *p*-MB, *p*-methylbenzyl; TFA, trifluoroacetic acid.

(2) Lord, J. A.; Waterfield, A. A.; Hughes, J.; Kosterlitz, H. W. *Nature (London)* **1977**, *267*, 495–499.
(3) Wolozin, B. L.; Pasternak, G. W. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 6181–6185.
(4) Martin, W. R.; Easdes, C. G.; Thompson, J. A.; Huppler, R. E.; Hilbert, P. E. *J. Pharmacol. Exp. Ther.* **1976**, *197*, 517–524.
(5) For a recent comprehensive review, see: Hruby, V. J.; Gehrig, C. A. *Med. Res. Rev.* **1989**, *9*, 343–401.
(6) Hruby, V. J. *Life Sci.* **1982**, *31*, 189–199.
(7) Hruby, V. J.; Al-Obeidi, F.; Kazmierski, W. *Biochem. J.* **1990**, *268*, 249–262.
(8) Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Gee, K.; Yamamura, H. I.; Galligan, J. J.; Burks, T. F. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5871–5874.
(9) Hruby, V. J.; Kao, L.-F.; Hirning, L. D.; Burks, T. F. In *Peptides: Structure and Function*; Deber, C. M., Hruby, V. J., Kopple, K. D., Eds.; Pierce Chemical Co.: Rockford, IL, 1985; pp 487–490.
(10) Toth, G.; Kramer, T. H.; Knapp, R.; Lui, G.; Davis, P.; Burks, T. F.; Yamamura, H. I.; Hruby, V. J. *J. Med. Chem.* **1990**, *33*, 249–253.
(11) Pelton, J. T.; Gulya, K.; Hruby, V. J.; Duckles, S. P.; Yamamura, H. I. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 236–239.
(12) Kazmierski, W.; Hruby, V. J. *Tetrahedron* **1988**, *44*, 697–710.
(13) Kazmierski, W.; Wire, S. W.; Lui, G. K.; Knapp, R. J.; Shook, J. E.; Burks, T. F.; Yamamura, H. I.; Hruby, V. J. *J. Med. Chem.* **1988**, *31*, 2170–2177.
(14) Kawasaki, A.; Knapp, R. J.; Kramer, T. H.; Wire, W. S.; Vasquez, O. S.; Yamamura, H. I.; Burks, T. F.; Hruby, V. J. *J. Med. Chem.* **1990**, *33*, 1874–1879.

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para position of the aromatic side-chain groups of Phe[4]. Some of these analogues,[10] especially the halogenated analogues such as [p-ClPhe[4]]DPDPE and [p-IPhe[4]]-DPDPE were substantially more potent (5–10-fold) and more δ opioid receptor selective (5–10-fold) than the parent DPDPE, providing convincing evidence of the importance of the Phe[4] residue for δ opioid receptor binding and selectivity. Preliminary nuclear magnetic resonance spectroscopy studies[9] indicated that no significant perturbation of the backbone conformation occurred as a result of these substitutions, consistent with predictions from the proposed conformational model.[17] Recently a new series of linear peptides from frog skin with exceptional potency and selectivity for δ opioid receptors as measured in particular by K_i values in rat brain membrane binding assays has been discovered.[18,19] These compounds, which are referred to as deltorphins or dermenkephalins, provide an important new lead for understanding the δ opioid receptor.

Examination of the topographical requirements for interaction of a peptide with its receptor(s) constitutes another important area for peptide design and for investigation of conformation–biological activity relations.[7,12] Such investigations are best utilized in the context of a conformationally constrained peptide with a reasonably stable conformation. In this approach one seeks to bias or constrain a side-chain moiety of a residue in a peptide to a particular side chain conformation (e.g. gauche(−), Figure 1A) while the overall backbone conformation is maintained. In this manner, the topographical relationship of side-chain groups to one another are modified, creating different surface characteristics which will interact differently with different potential receptors for the peptide. In this manner, increased potency and/or selectivity at a particular receptor can be anticipated. We have applied these principles to the design of highly selective ligands for the μ opioid receptor[12,13] and to oxytocin antagonists.[20,21] In this paper, we report on the use of β-methylphenylalanine and β-methyl-p-nitrophenylalanine analogues of DPDPE for topographical design of δ opioid ligands. The opioid receptor affinities and selectivities were evaluated in the MVD and GPI bioassays and in rat brain binding assays in comparison with the parent ligands DPDPE. Very large differences in binding and selectivities were observed, suggesting that such a design approach may provide a powerful approach in peptide ligand design. A preliminary report has appeared for some of this work.[22,23]

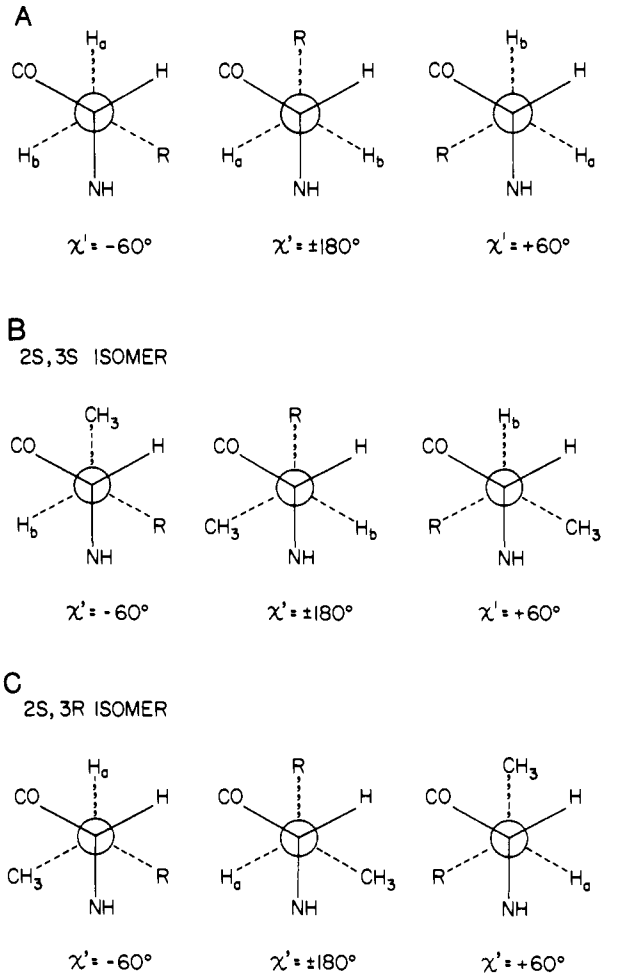

Figure 1. (A) Side-chain rotamer populations for α-amino acids about the χ_1 torsional angle, (B) Newman projection of the side-chain conformations of (2S,3S)-β-methylphenylalanine where R is equal to phenyl, (C) Newman projection of the side-chain conformations of (2S,3R)-β-methylphenylalanine where R is equal to phenyl.

Results

The synthesis of the β-MePhe amino acid were first prepared[24] in their erythro (D,L) and threo (D,L) forms and incorporated into DPDPE in the Phe[4] position as their enantiomeric pairs (subsequently the identity of the amino acids were confirmed by comparison with the enantiomerically pure amino acids prepared by asymmetric synthesis[25]). The *erythro*-D,L- and *threo*-D,L-β-methyl-p-nitrophenylalanines were prepared directly from the corresponding β-methylphenylalanines by direct electrophilic aromatic substitution followed by purification (see the Experimental Section for details). N^{α}-Boc-protected enantiomeric amino acids were used directly for the synthesis of the peptide precursors.

All of the new analogues of DPDPE were prepared by the solid-phase method of peptide synthesis by using methods very similar to those used previously in the synthesis of DPDPE and Phe[4]-substituted analogues.[8,10] The diastereoisomeric products generally were purified and separated by gel filtration on a Sephadex G-10 column followed by reversed-phase high-performance liquid chromatography (RP-HPLC) (see the Experimental Sec-

(15) James, I. F.; Goldstein, A. *Mol. Pharmacol.* **1984**, *25*, 337–341.
(16) Akiyama, K.; Gee, K. W.; Mosberg, H. I.; Hruby, V. J.; Yamamura, H. I. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 2543–2547.
(17) Hruby, V. J.; Kao, L.-F.; Pettitt, B. M.; Karplus, M. *J. Am. Chem. Soc.* **1988**, *110*, 3351–3359.
(18) Erspamer, V.; Melchiorri, P.; Falconieri-Erspamer, G.; Negri, L.; Lorsi, R.; Severini, C.; Barra, D.; Simmaco, M.; Kreil, G. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5188–5192.
(19) Kreil, G.; Barra, D.; Simmaco, M.; Erspamer, V.; Falconieri-Erspamer, G.; Negri, L.; Severini, C.; Corsi, R.; Melchiorri, P. *Eur. J. Pharmacol.* **1989**, *162*, 123–128.
(20) Hill, P. S.; Smith, D. D.; Slaninova, J.; Hruby, V. J. *J. Am. Chem. Soc.* **1990**, *112*, 3110–3113.
(21) Lebl, M.; Hill, P.; Kazmierski, W.; Karaszova, L.; Slaninova, J.; Fric, I.; Hruby, V. J. *Int. J. Pept. Protein Res.* **1990**, *36*, 321–330.
(22) Hruby, V. J.; Toth, G.; Prakash, O.; Davis, P.; Burks, T. F. In *Peptides 1988, Proceedings of the 20th European Peptide Symposium*; Jung, G., Bayer, E., Eds.; W. de Gruyter & Co.: Berlin, 1989; pp 616–618.
(23) Kramer, T. H.; Lui, G. K.; Yamamura, H. I.; Toth, G.; Hruby, V. J.; Burks, T. F. In *Progress in Opioid Research*; Advances in Biosciences; Cros, J., Meunier, J.-Cl., Hamon, M., Eds.; Pergamon Press: Oxford, 1989; Vol. 75, pp 101–104.
(24) Kataoka, Y.; Seto, Y.; Yamamoto, M.; Kuwata, S.; Watanabe, H. *Bull. Chem. Soc. Jpn.* **1976**, *49*, 1081–1084.
(25) Dharanipragada, R.; Nicolas, E.; Toth, G.; Hruby, V. J. *Tetrahedron Lett.* **1989**, *30* (49), 6841–6844.

Table I. DPDPE Analogues Inhibitory Potency and Selectivity in MVD and GPI Bioassays

peptide	MVD[a] IC_{50}, nM	GPI[a] IC_{50}, nM	GPI[a] $\%E_{max}$[b]	selectivity[c] ratio
1, Tyr-D-Pen-Gly-Phe-D-Pen-OH (DPDPE)	4.1 ± 0.46	7300 ± 1700	90	1810
2, Tyr-D-Pen-Gly-(2*S*,3*S*)-β-MePhe-D-Pen-OH	39 ± 3.9	57400 ± 17600	90	1500
3, Tyr-D-Pen-Gly-(2*R*,3*R*)-β-MePhe-D-Pen-OH	27500 ± 390	48300 ± 5500	100	18
4, Tyr-D-Pen-Gly-(2*S*,3*R*)-β-MePhe-D-Pen-OH	166 ± 25	3200 ± 1900	56	>47
5, Tyr-D-Pen-Gly-(2*R*,3*S*)-β-MePhe-D-Pen-OH	38000 ± 2700	166000 ± 14000	100	4.4
6, Tyr-D-Pen-Gly-(2*S*,3*S*)-β-Me-*p*-NO_2Phe-D-Pen-OH	4.59 ± 0.21	25700 ± 4500	73	8800
7, Tyr-D-Pen-Gly-(2*R*,3*R*)-β-Me-*p*-NO_2Phe-D-Pen-OH	630 ± 81	>10000	NA[d]	>20
8, Tyr-D-Pen-Gly-(2*S*,3*R*)-β-Me-*p*-NO_2Phe-D-Pen-OH	27 ± 2.3	2700 ± 550	74	150
9, Tyr-D-Pen-Gly-(2*R*,3*S*)-β-Me-*p*-NO_2Phe-D-Pen-OH	1790 ± 190	12300 ± 2700	66	13

[a] MVD = mouse vas deference assay; GPI = guinea pig ileum assay; n = 2–4 experiments in duplicate. [b] The drug effects are expressed as absolute percent inhibition of contraction according to the expression % inhibition = 100 × control − test/control, where control is the contraction measured in the absence of analogue and test is the contraction strength after addition of analogue. The mean data were subjected to nonlinear regression analysis to obtain the maximal inhibition (E_{max}) and potency represented by the IC_{50} (concentration of drug producing 50% or one-half of E_{max}). [c] The selectivity ratio represents the ratio of concentrations required to produce *equal* degrees of inhibition in the GPI and MVD. It should be noted that the simple ratio of IC_{50} in the GPI and MVD would give a different (misleading) result in many cases as the IC_{50} value is dependent on the E_{max} in the GPI which varied considerably for the analogues examined in the GPI. [d] Not applicable.

tion for complete details for each compound). As can be seen in Table IV, all four isomers of β-MePhe⁴-substituted DPDPE (**2–5**) and all four isomers of β-methyl-*p*-nitrophenylalanine (β-Me-*p*-NO_2Phe) substituted DPDPE (**6–9**) were obtained in a highly purified form (see the Experimental Section for details).

The potencies of the synthesized DPDPE analogues to inhibit electrically evoked contractions of the myenteric plexus longitudinal muscle of the guinea pig ileum (GPI) and of the mouse vas deferens (MVD) are summarized in Table I. Results obtained for DPDPE are included for comparison. The β-MePhe⁴ analogues (**2–5**) and the β-Me-*p*-NO_2Phe⁴ analogues (**6–9**) behave similarly, but there are some differences and hence the results will be examined separately. As shown in Table I, the substitution of a β-MePhe for Phe⁴ in DPDPE has a large effect on the bioactivities particularly in the MVD, but also in the GPI bioassays depending on the isomer used. When the analogues containing the L-amino acids (2*S*,3*S*)-β-MePhe (**2**) and (2*S*,3*R*)-β-MePhe (**4**) are compared to DPDPE, compounds with high δ opioid receptor selectivity are obtained. Particularly noteworthy is the greatly reduced E_{max} (maximal inhibition) of the 2*S*,3*R* compound **4** in the GPI (μ receptor) assay, suggesting it is a weak partial agonist in this assay. Both D-amino acid containing analogues [(2*R*,3*R*)-β-MePhe⁴]DPDPE (**3**) and [(2*R*,3*S*)-β-MePhe⁴]DPDPE (**5**) show very weak potencies at both receptors. Analogue **3** and the previously prepared [D-Phe⁴]DPDPE are very weak, slightly δ selective ligands in these assays. On the other hand, the D-amino acid containing analogue **5** appears to be virtually inactive at the δ opioid receptor as measured in the MVD assay as well as the μ opioid receptor as measured by the GPI assay. It is clear that the topographical features at position 4 can have a dramatic effect on the interaction of DPDPE with the δ receptor. Also, the interaction with the μ receptor is decreased for all four analogues relative to DPDPE.

The situation is similar, but slightly different, for the β-Me-*p*-NO_2Phe⁴ analogues of DPDPE (**6–9**) (Table I). In all cases, large differences in potency and selectivity for the δ vs μ opioid receptor are observed. In these cases, the L-amino acid analogues **6** and **8** are much more potent and selective in the MVD (δ) opioid receptor assays than the D-amino acid analogues **7** and **9**. Interestingly, [(2*S*,3*S*)-β-Me-*p*-NO_2Phe⁴]DPDPE (**6**) is considerably more selective than DPDPE as measured in these bioassays, because of a 4-fold decrease in potency in the GPI assay relative to DPDPE, whereas, in the 2*S*,3*R* analogue **8**, a 6-fold decrease in the MVD assay is observed and a 2.7-fold decrease in potency is seen in the GPI assay relative to DPDPE, making the latter analogue much less selective. Both the D-amino acid analogues **7** and **9** are much less potent in the δ assay (MVD) than DPDPE by factors of about 120 and 350, respectively. They retain some δ opioid receptor selectivity in these in vitro assays, however, due to the very weak potency of both in the GPI (μ) receptor assay.

Similar trends were observed in the rat brain membrane binding assays (Table II) in that there are large differences in potencies and selectivities for the β-MePhe⁴ and the β-Me-*p*-NO_2Phe⁴ analogues of DPDPE. The highly δ opioid selective ligands [³H]DPDPE[8,16] or [³H][*p*-ClPhe⁴]DPDPE[10,26] were used to assess binding to the δ opioid receptor, and [³H]D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2)[11,27] ([³H]CTOP) was used to assess binding to μ opioid receptor in the rat brain. In both the β-MePhe⁴ and the β-Me-*p*-NO_2Phe⁴ analogues, the L-amino acid containing diastereoisomers 2*S*,3*S* and 2*S*,3*R* (**2**, **4**, **6**, and **8**, respectively) are more potent and more selective for the δ opioid receptor than the corresponding D-amino acid containing diastereoisomers 2*R*,3*R* and 2*R*,3*S* (**3**, **5**, **7**, and **9**, respectively). Particularly noteworthy is the very high binding selectivity of the analogue [(2*S*,3*S*)-β-MePhe⁴]DPDPE (**2**). This compound is about 1,800-fold selective in binding to the δ vs μ receptor as assessed by these competitive binding assay. This makes it the most selective δ opioid receptor ligands in this series as assessed by rat brain binding assays using highly μ and δ opioid receptor selected radiolabeled ligands, respectively. It is interesting to note that both the (2*S*,3*R*)-β-MePhe-containing analogue **4** and the (2*S*,3*R*)-β-Me-*p*-NO_2Phe⁴-containing analogue **6** are also more selective than DPDPE for the δ opioid receptor. They are more selective for different reasons, however. Compound **6** is 10 times more potent than **4** in binding to the δ opioid

(26) Vaughn, L. K.; Knapp, R. J.; Toth, G.; Wan, Y.-P.; Hruby, V. J.; Yamamura, H. I. *Life Sci.* **1989**, *45*, 1001–1008.
(27) Hawkins, K. N.; Knapp, R. J.; Lui, G. K.; Gulya, K.; Kazmierski, W.; Wan, Y.-P.; Pelton, J. T.; Hruby, V. J.; Yamamura, H. I. *J. Pharmacol. Exp. Ther.* **1989**, *248*, 73–81.

Table II. DPDPE Analogues Binding Affinities and Selectivity in Competition with [^{3}H]CTOP, [^{3}H]DPDPE, or [^{3}H][*p*-ClPhe^4]DPDPE in Rat Brain Receptor Binding Assays

peptide	[^{3}H]DPDPE[a,b]		[^{3}H]CTOP[a]		ratio[c]
	IC_{50}, nM	K_i, nM	IC_{50}, nM	K_i, nM	
1, Tyr-D-Pen-Gly-Phe-D-Pen-OH (DPDPE)	5.83 ± 0.89		619 ± 280		116
2, Tyr-D-Pen-Gly-(2*S*,3*S*)-β-MePhe-D-Pen-OH	38 ± 4.0	10 ± 1.5	69000 ± 8900	14000 ± 3700	1800
3, Tyr-D-Pen-Gly-(2*R*,3*R*)-β-MePhe-D-Pen-OH	1420 ± 324	390 ± 1101	73000 ± 12000	>16200	51
4, Tyr-D-Pen-Gly-(2*S*,3*R*)-β-MePhe-D-Pen-OH	62.7 ± 260	15 ± 1.5	33500 ± 12500	1700 ± 910	530
5, Tyr-D-Pen-Gly-(2*R*,3*S*)-β-MePhe-D-Pen-OH	1660 ± 260	450 ± 55	36000 ± 12000	8200 ± 2200	22
6, Tyr-D-Pen-Gly-(2*S*,3*S*)-β-Me-*p*-NO_2Phe-D-Pen-OH	6.89 ± 0.83	5.5 ± 0.66	2200 ± 1200	530 ± 320	325
7, Tyr-D-Pen-Gly-(2*R*,3*R*)-β-Me-*p*-NO_2Phe-D-Pen-OH	226 ± 25	180 ± 20	>10000[d]	ND	>40
8, Tyr-D-Pen-Gly-(2*S*,3*R*)-β-Me-*p*-NO_2Phe-D-Pen-OH	4.2 ± 1.9	3.4 ± 1.5	105 ± 31	24 ± 7.5	25
9, Tyr-D-Pen-Gly-(2*R*,3*S*)-β-Me-*p*-NO_2Phe-D-Pen-OH	222 ± 23	180 ± 18	1018 ± 370	240 ± 90	4.5

[a] Values are reported as IC_{50} and K_i values ± standard error of the mean (SEM); n = 2–4 experiments in duplicate for these studies. [b] Values for K_i for analogues 2–5 were measured against [^{3}H][*p*-ClPhe^4]DPDPE. [c] The ratios given are from IC_{50} values. [d] 41% displacement at 10000. [e] Not determined.

receptor, but it actually is somewhat less selective due to its increased binding to the μ receptor (Table II). Most interesting is the high potency, but reduced selective, of the (2*S*,3*R*)-β-Me-*p*-NO_2Phe^4-containing analogue 8. It is the most potent ligand of the series in the binding assay, but one of the least selective due to its surprisingly potent binding to the μ opioid receptor. All of the analogues containing a D-amino acid in the 4-position have large reductions in binding to the δ opioid receptor (about 40–300 times less) relative to DPDPE. However, none are particularly selective due to weak binding to the μ opioid receptor as well. However, it is noteworthy that [(2*R*,3*S*)-β-Me-*p*-NO_2]DPDPE (9, Table II) displays micromolar binding to the μ opioid receptor. It should be further noted that the β-Me-*p*-NO_2Phe^4-containing analogues are generally less selective than the β-MePhe^4-containing analogues in rat brain binding. This is because the latter generally bind more weakly at the δ receptor than the former, and the former bind much better than the latter to the μ opioid receptor in the rat brain.

Discussion

The use of topographical constraints[6,7,9,12,28] in the design of biologically active peptides has considerable potential, in principle, for the design of peptides with specific surface characteristics and increasing selectivity and specificity in the design of peptide ligands for receptors, enzymes, and other biological systems where molecular recognition is important to biological activity and specificity.[7,12,13,20–22] Topographical design in the context of this paper is concerned with the relative, three-dimensional arrangements of the side-chain groups in a peptide ligand. More specifically, we are seeking to constrain or bias a side chain group to a particular χ_1 side-chain torsional angle within the context of a particular backbone conformation. In the case of DPDPE, we have decided to utilize substitution at the diastereotopic β-hydrogens of phenylalanine as a way of biasing the side-chain groups to particular side-chain torsional angles. For this purpose, β-methyl substitution was chosen. As shown in Figure 1B, simple steric considerations would suggest that for the L-amino acid analogue in which the *pro-S* hydrogen of Phe was substituted with a methyl group to give (2*S*,3*S*)-β-methylphenylalanine ((2*S*,3*S*)-β-MePhe), a gauche(−) conformation would be predicted to be preferred. Similarly, when the *pro-R* methyl substitution is made, to give (2*S*,3*R*)-β-methylphenylalanine, the trans side-chain conformation (Figure 1C) would be expected to be preferred. Similar considerations apply for the D-amino acids (2*R*,3*S*)-β-MePhe and (2*R*,3*R*)-β-MePhe except, of course, that the relationship of the $CH_2C_6H_5$ side chain to the rest of the peptide is now enantiomeric, leading to additional topographical changes when incorporated into a peptide.

With these thoughts in mind, and with the recognition from previous studies that the Phe^4 residue in DPDPE was important for δ opioid receptor interactions, we have synthesized all four diastereoisomeric peptides of DPDPE with (2*S*,3*S*)-, (2*R*,3*R*)-, (2*S*,3*R*)-, and (2*R*,3*R*)-β-methylphenylalanine in position 4. All four diastereoisomeric analogues with the corresponding β-methyl-*p*-nitrophenylalanine residues in position 4 also were synthesized. All eight isomers were examined for their activity in the GPI and MVD in vitro bioassays (Table I) and in rat brain binding displacement assays (Table II) with highly opioid receptor selective radiolabeled ligands. The results in Tables I and II provide interesting results and illustrate the potential power of the topographical approach in the design of biologically active peptides. The large differences in potency and selectivity at both μ and especially δ receptors in the MVD and GPI assays, respectively, suggest that the δ opioid receptor in particular has specific topographical requirements for the Phe^4 position in the cyclic DPDPE series.

In this regard, it is interesting to note that in the highly δ selective deltorphin compounds,[18,19] the Phe residue is found in the 3-position rather than in the 4-position as found in the cyclic enkephalin series of DPDPE analogues. This raises the interesting question about whether the Phe^3 residue in the deltorphins plays an equally important topographical role in the remarkable δ opioid receptor selectivity of these ligands as it does in the DPDPE analogues. We plan to address this issue in the future by examining the β-MePhe^3-substituted analogues of the deltorphins. In the meantime, we have addressed this issue by energy calculations on deltorphins and compared the results obtained with those obtained for DPDPE.[29] The results suggest[29] that though the deltorphins have quite different conformational preferences than DPDPE, examination of their comparative topographical properties indicate that the Phe^4 aromatic moiety in DPDPE and the Phe^3 aromatic moiety in deltorphin can occupy essentially

(28) Kazmierski, W.; Yamamura, H. I.; Burks, T. F.; Hruby, V. J. In *Peptides 1988, Proceedings of the 20th European Peptide Symposium*; W. de Gruyter & Co.: Berlin, 1989; pp 643–645.

(29) Nikiforovich, G. V.; Hruby, V. J. *Biochem. Biophys. Res. Commun.* **1990**, *173*, 521–527.

Table III. Melting Point and R_f Values for β-Methyl Amino Acids and Their Derivatives

	mp, °C	R_f A	R_f B	R_f C	R_f chiral TLC
erythro-β-methyl-Phe	224	0.48	0.43		0.56 0.36
threo-β-methyl-Phe	218	0.48	0.43		0.55 0.47
erythro-β-methyl-*p*-nitro-Phe	193	0.50	0.51		0.62 0.43
threo-β-methyl-*p*-nitro-Phe	188	0.500	0.51		0.60 0.52
(*S,S*)-β-methyl-*p*-nitro-Phe	200	0.50	0.51		0.62
(*R,R*)-β-methyl-*p*-nitro-Phe	186	0.50	0.51		0.43
N^{α}-Boc-*erythro*-β-methyl-Phe	108		0.82	0.69	
N^{α}-Boc-*threo*-β-methyl-Phe	96–96		0.82	0.69	
N^{α}-Boc-*erythro*-β-methyl-*p*-nitro-Phe	148–149		0.78	0.58	
N^{α}-Boc-*threo*-β-methyl-*p*-nitro-Phe	84–86		0.78	0.58	

the same three-dimensional space. Further studies with topographically constrained deltorphins and the highly δ selective linear enkephalin analogues of Roques and coworkers[30] should lead to further insights into the topographical requirements of the δ opioid receptor.

The analogues of DPDPE reported in this paper are all weakly potent in the GPI assay, but there are significant differences depending on the isomer, suggesting that the Phe⁴ residue can modulate potency at the μ receptor to some extent. The [(2*S*,3*R*)-β-MePhe⁴]DPDPE analogue 4 is 4-fold less potent than [(2*S*,3*S*)-β-MePhe⁴]DPDPE analogue 2 in the MVD (δ receptor) assay suggesting that the gauche(−) conformation for the β-MePhe⁴ is preferred by the δ receptor relative to the trans side-chain rotamer. Nonetheless, both 2 and 4 are fully active agonists in the MVD assay and remain highly selective. Thus, especially 2 due to its high selectivity would appear to be an excellent analogues for examination of the physiological and pharmacological roles of δ opioid receptors in peripheral tissue.

This appears to be especially true in the brain as well, on the basis of the rat brain binding assay. Particularly interesting is the very high selectivity of [(2*S*,3*S*)-β-MePhe⁴]DPDPE relative to DPDPE (about 12–15 times more selective). Though this analogue appears to be less potent than DPDPE in the rat brain binding assay than in the in vitro MVD bioassay, it nonetheless should be a useful ligand for in vitro and in vivo studies of the physiological and pharmacological roles of δ receptors in the brain because of its high selectivity. These results also suggest that there are significant differences in the structural and perhaps topographical requirements for the δ receptors in the brain and in peripheral tissues. For example, [(2*S*,3*R*)-β-Me-*p*-NO_2Phe⁴]DPDPE 8 is nearly 5-fold less potent than [(2*S*,3*S*)-β-Me-*p*-NO_2Phe⁴]DPDPE in the MVD assay, but is nearly 2-fold more potent in the binding studies in the rat brain. While other explanations such as differences in distribution or receptor availability in the two different assays may be invoked as possible explanations for these differences, the simpler explanation of different receptor requirements seems more plausible in these simple assays. Furthermore, there is now significant other evidence for δ opioid receptor heterogeneity.[31,32] These new ligands, therefore, may serve as new tools to examine this possibility. Other peptide ligands that would appear to have considerable interest in this regard include the deltorphins[18,19] and highly selective analogues,[33] [D-Ala²,Leu⁵,Cys⁶]enkephalin,[34] and [D-Ser(O^{t}Bu)²,Leu⁵,Thr(O^{t}Bu)⁶]enkephalin.[30]

Finally, it has not escaped our notice that many peptide hormones and neurotransmitters have key aromatic amino acid residues important to their receptor binding and transduction mechanisms. The use of topographically biased amino acid residues such as β-methylphenylalanine, β-methyltyrosine, etc., should provide a powerful tool for the design of other peptide ligands for receptors, enzymes, antibodies, and other biological acceptors. They should also provide new insights into the structural modifications that are compatible with design of specific conformational and topographical features in peptides.

Experimental Section

General Methods for β-Methyl Amino Acids. TLC was performed on 2.5 × 10 cm plates coated with silica gel GF (Analtech) using the following systems: A, butanol–acetic acid–water (4:1:1); B, acetonitrile–methanol–water (4:1:1); C, Chloroform–methanol–acetic acid (90:8:2). TLC spots were detected under UV light (short wavelength) and with ninhydrin spray. Chiral TLC was performed on chiral plates (Macherey-Nagel) using system B by methods similar to those previously used in our laboratory.[35]

Melting points were measured by a Thomas-Hoover capillary melting point apparatus and are uncorrected. NMR spectra were obtained on a Bruker WM-250 250-MHz spectrometer equipped with an ASPECT 2000 computer. Spectra were obtained in either $CDCl_3$, D_2O, or D_2O/TFA. Mass spectra were obtained at the University of Arizona Microanalysis Center using chemical ionization with isobutane. Optical rotations were measured on a Perkin-Elmer 141 polarimeter.

Synthesis of β-Methylphenylalanine and *erythro*-β-Methylphenylalanine and *threo*-β-Methylphenylalanine. β-Methylphenylalanine was prepared by a slightly modified method of Kataoka et al.[24] The pure *erythro*-(2*S*,3*S*)- and -(2*R*,3*R*)-β-methylphenylalanine hydrochloride salt could be obtained by fractional crystallization from water. The free amino acid was liberated with diluted NH_4OH by adjusting the pH to 5.6 with a pH-stat. The *threo*-(2*S*,3*R*)- and (2*R*,3*S*)-β-methylphenylalanine was obtained from the mother liquor by repeated crystallization from water. *erythro*-β-Methylphenylalanine: NMR (D_2O) δ 7.27 (5 H, aromatic), 3.64 (1 H, H_α, d, J = 7.7 Hz), 3.15 (1 H, H_β, q, J = 7.33 Hz), 1.27 (3 H, CH_3, d, J = 7.15 Hz). *threo*-β-methylphenylalanine: NMR (D_2O) δ 7.16 (5 H, aromatic), 3.71 (1 H, H_α, d, J = 4.95 Hz), 3.31 (1 H, H_β, q, J = 7.25 Hz), 1.27 (3 H, CH_3, d, J = 7.31). Melting points and R_f values are in Table III.

Synthesis of *erythro*- and *threo*-β-Methyl-*p*-nitrophenylalanines. *erythro*- or *threo*-β-methylphenylalanine (3.6 g) was dissolved in 5 mL of concentrated sulfuric acid (1.73 mL)

(30) Delay-Goyet, P.; Seguin, C.; Gacel, G.; Roques, B. P. *J. Biol. Chem.* **1988**, *263*, 4124–4130.
(31) Takamori, A. E.; Sofuoglu, M.; Sultana, M.; Nagase, H.; Portoghese, P. S. In *New Leads in Opioid Research*, van Ree, J. M., Mulder, A. H., Wiegand, V. M., van Wimersma Greidanus, T. B., Eds.; Excerpta Medica: Amsterdam, 1990; pp 277–278 and references therein.
(32) Porreca, F. In *New Leads in Opioid Research*; Excerpta Medica: Amsterdam, 1990; pp 280–282 and references therein.
(33) Schiller, P. W.; Nguyen, T. M.-D.; Weltrowska, G.; Lemieux, C.; Chung, N. N. In *New Leads in Opioid Research*; Excerpta Medica: Amsterdam, 1990; pp 288–290.
(34) Bowen, W. D.; Hellewell, S. B.; Kelemen, M.; Huey, R.; Stewart, D. *J. Biol. Chem.* **1987**, *262*, 13434–13439.
(35) Toth, G.; Lebl, M.; Hruby, V. J. *J. Chromatogr.* **1990**, *504*, 450–455.

Table IV. Analytical Properties of Synthetic Peptide Analogues

peptide	TLC[a] A	TLC[a] B	TLC[a] C	TLC[a] D	HPLC[b]	FAB-MS (M + 1)$^+$ calcd	FAB-MS (M + 1)$^+$ found
1, Tyr-D-Pen-Gly-Phe-D-Pen-OH (DPDPE)	0.43	0.66	0.86	0.81	1.85	646	646
2, Tyr-D-Pen-Gly-(2*S*,3*S*)-β-MePhe-D-Pen-OH	0.55	0.79	0.85	0.84	2.04	660	660
3, Tyr-D-Pen-Gly-(2*R*,3*R*)-β-MePhe-D-Pen-OH	0.58	0.69	0.78	0.84	3.23	660	660
4, Tyr-D-Pen-Gly-(2*S*,3*R*)-β-MePhe-D-Pen-OH	0.57	0.79	0.82	0.84	2.53	660	660
5, Tyr-D-Pen-Gly-(2*R*,3*S*)-β-MePhe-D-Pen-OH	0.59	0.74	0.78	0.82	3.24	660	660
6, Tyr-D-Pen-Gly-(2*S*,3*S*)-β-Me-*p*-NO_2Phe-D-Pen-OH	0.46	0.64	0.91	0.82	3.32	705	705
7, Tyr-D-Pen-Gly-(2*R*,3*R*)-β-Me-*p*-NO_2Phe-D-Pen-OH	0.41	0.61	0.81	0.82	7.67	705	705
8, Tyr-D-Pen-Gly-(2*S*,3*R*)-β-Me-*p*-NO_2Phe-D-Pen-OH	0.48	0.53	0.81	0.75	3.98	705	705
9, Tyr-D-Pen-Gly-(2*R*,3*S*)-β-Me-*p*-NO_2Phe-D-Pen-OH	0.48	0.53	0.77	0.75	5.37	705	705

[a] Merck DC-Fetigplatten Kieselgel 90F_{25b} 0.25 mm analytical silica gel plates were used. Solvent systems: A, butanol–acetic acid–water 4:1:1; B, butanol–acetic acid–pyridine–water 13:3:12:10; C, 2-propanol–ammonia–water 3:1:1; D, butanol–acetic acid–ethyl acetate–water 1:1:1:1. [b] Capacity factor for Vydac 218TP104 C_{18} reversed phase column (25 × 0.46 cm) with 0.1% trifluoroacetic acid–acetonitrile (75/25 v/v). Flow rate 1.5 mL/min. All peptides were monitored at λ = 214 and 280 mm.

and 1.3 equiv of 70% of nitric acid was added to the syrup at 0 °C dropwise with stirring. The mixture was stirred for 30 min at room temperature and then poured into 75 mL of cold water. The pH of the solution was adjusted to 5.6. The crystals were filtered to yield 3.1 g (70%) of D,L-*erythro*-β-methyl-*p*-nitrophenylalanine or 2.2 g (50%) of D,L-*threo*-β-methyl-*p*-nitrophenylalanine. *erythro*-β-Methyl-*p*-nitrophenylalanine: NMR (D_2O, TFA) δ 7.98 (2 H, aromatic ortho to nitro, d, *J* = 8.74 Hz), 7.31 (2 H, aromatic meta to nitro, d, *J* = 8.73 Hz), 4.015 (1 H, H_α, d, *J* = 6.92 Hz), 3.35 (1 H, H_β, q, *J* = 7.08 Hz), 1.17 (3 H, CH_3, d, *J* = 7.15). *threo*-β-Methyl-*p*-nitrophenylalanine: NMR (D_2O, TFA) δ 7.94 (2 H, aromatic ortho to nitro, d, *J* = 8.75 Hz), 7.28 (2 H, aromatic meta to nitro, d, *J* = 8.75 Hz), 4.05 (1 H, H_α, d, *J* = 6.23), 3.38 (1 H, H_β, q, *J* = 6.84 Hz), 1.22 (3 H, CH_3, d, *J* = 7.2 Hz). The (M + 1) molecular ions were 225 for both compounds in agreement with the calculated molecular weights. Melting points and R_f values are in Table III.

erythro-β-Methyl-*p*-nitro-L-phenylalanine or *erythro*-β-methyl-*p*-nitro-D-phenylalanine were also prepared by the above methodology starting with pure isomers of *erythro*-β-methylphenylalanine. Optical rotation for *erythro*-β-methyl-*p*-nitro-L-phenylalanine: $[\alpha]^{20}_D$ = −14.7° (*c* 0.8, H_2O). For *erythro*-β-methyl-*p*-nitro-D-phenylalanine: $[\alpha]^{20}_D$ = +14.6° (*c* 0.8, H_2O).

Synthesis of the N^α-*tert*-Butyloxycarbonyl Derivatives of β-Methyl-*p*-nitrophenylalanines. Synthesis of the N^α-(*tert*-butyloxycarbonyl)-β-methyl amino acids were carried out by slight modifications of published procedures[36] as illustrated below. A solution of 2.24 g (10 mmol) of *erythro*-β-methyl-*p*-nitrophenylalanine *or* 2.24 (10 mmol) of *threo*-β-methyl-*p*-nitrophenylalanine in 70 mL of dioxane–water (2:1) was cooled in an ice bath, and 2.4 g (12 mmol) of di-*tert*-butyl dicarbonate was added. The pH was adjusted to 10.2 with 4 N sodium hydroxide, and the pH of the stirred solution was continuously adjusted to 10.2. When the pH did not change further, the mixture was stirred at room temperature overnight. The solvents were removed under vacuum, and the solid was dissolved in water and cooled to 0 °C, and 50 mL of ethyl acetate was added. Then 10% aqueous citric acid was added until an acidic aqueous solution was obtained. The aqueous layer was extracted with 3 × 50-mL portions of ethyl acetate. The combined organic phases were washed with water, brine, and water (50 mL), and the organic phase was dried over anhydrous Na_2SO_4. The solvent was evaporated and the residue dissolved in a minimum amount of ethyl acetate. Petroleum ether was then slowly added to give white crystalline products: 2.27 g (70%) of N^α-Boc-*erythro*-β-methyl-*p*-nitrophenylalanine and 2.11 g (65%) of N^α-Boc-*threo*-β-methyl-*p*-nitrophenylalanine. The analytical data are given in Table III.

General Methods for the Synthesis of DPDPE Analogues. All of the peptide analogues of DPDPE were synthesized by solid-phase peptide methods using procedures similar to those previously reported for DPDPE.[8] Chloromethylated (1.3 mmol/g resin) polystyrene resin 1% cross-linked with divinylbenzene (Lab Systems, San Mateo, CA) was used as the solid support. N^α-*tert*-Butyloxycarbonyl (Boc) protected amino acids were used throughout. The unprotected amino acids (Tyr and Gly) were obtained from Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO) and were converted to their N^α-*tert*-butyloxycarbonyl derivatives with di-*tert*-butyl dicarbonate (Fluka, Switzerland) following literature procedures.[37] The N^α-Boc-D-Pen(*S*-*p*-MeBzl) was obtained as the DCHA salt (Peptides International, Louisville, KY) and liberated just prior to use. The N^α-(*tert*-butyloxycarbonyl)-*S*-(*p*-methylbenzyl)-D-penicillamine was attached to the resin by Gysin's method.[38] Diisopropylcarbodiimide and hydroxybenzyltriazole (Aldrich, Milwaukee, WI) were used in the coupling reactions which were monitored by the ninhydrin test.[39] The peptides were cleaved from the resin with anhydrous HF (5 mL/g resin) with anisole added as a scavenger (2 mL/g resin) for 69 min at 0 °C. The peptide was extracted from the resin by first washing (3 × 10 mL) with anhydrous ethyl ether and then stirring of the resin under N_2 suspended in 40 mL of glacial acetic acid. The resin was then washed with 3 × 50 mL of 30% acetic acid, and lastly with 3 × 50 mL distilled water. The acetic acid and water filtrates were combined, frozen with dry ice/2-propanol, and lyophilized. The linear peptide was then cyclized with a 0.1 N solution of $K_3[Fe(CN)_6]$ according to published procedures.[8] The analogues were purified by gel filtration on Sephadex G-10 using 15% acetic acid and by HPLC (Perkin-Elmer or Spectra Physics) on a Vydac 218TP1010 C_{18} reversed-phase column (25 cm × 1 cm) using a linear gradient of 20%–35% CH_3CN in 0.1% aqueous trifluoroacetic acid, 1%/min at a flow rate of 4 mL/min, with UV detection at 214 or 280 nm (see details in Table IV).

Purity was determined by TLC in four solvent systems on silica gel and by analytical HPLC. Amino acid analyses were performed on a Beckman 120C amino acid analyzer or a Model 420 A amino acid analyzer (ABI). The unusual amino acid D-Pen was not determined. The (M + 1)$^+$ molecular ions and fragmentation patterns were obtained by FAB-MS and were in agreement with the calculated molecular weights for each peptide. ^1H NMR spectra were obtained for each analogue and were consistent with the amino acid sequence and structure of the peptides.

[D-Pen^2,D-Pen^5]enkephalin (DPDPE, 1). The title compound was prepared by the methods described above and was found to be identical with the compound previously synthesized.[8]

[D-Pen^2,(2*S*,3*S*)-β-MePhe^4,D-Pen^5]enkephalin (2) and [D-Pen^2,(2*R*,3*R*)-β-MePhe^4,D-Pen^5]enkephalin (3). The title compounds were prepared by the solid-phase method outlined

(36) Perses, A.; Piani, S.; de Castiglione, R. *Int. J. Pep. Protein Res.* **1983**, *21*, 227–233.

(37) Moroder, L.; Hallett, A.; Wünsch, E.; Keller, O.; Wersin, G. *Hoppe-Seyler Z. Physiol. Chem.* **1976**, *357*, 1651–1653.

(38) Gysin, B. F. *Helv. Chim. Acta* **1973**, *56*, 1476–1482.

(39) Kaiser, F.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem.* **1970**, *34*, 595–598.

above, starting with 2.94 g of N^{α}-Boc-D-Pen(*S*-*p*-MeBzl)-O-resin (substitution = 0.72 mmol/g of resin) (2.0 mmol), and the following protected amino acids were added in a stepwise fashion to the growing peptide chain: N^{α}-Boc-*erythro*-D,L-β-MePhe, N^{α}-Boc-Gly, N^{α}-Boc-D-Pen(*S*-*p*-MeBzl), and N^{α}-Boc-Tyr. Then 2.2 g of the resulting 4.41 g of peptide resin was treated with 15 mL of HF along with 1.5 mL of anisole for 60 min at 0 °C. The HF was then rapidly removed by vacuum aspiration at 0 °C. The mixture was washed with 3 × 10 mL of ethyl ether, and the peptide was extracted with 40 mL of glacial acetic acid and washed with 5 × 50 mL of 30% acetic acid and then 5 × 50 mL of distilled water. The peptide solution was frozen with dry ice/2-propanol and lyophilized. The residue was dissolved in 1500 mL of deaerated, distilled water (using 3 mL of methanol to dissolve completely). The pH was adjusted to 8.5 with 3 N ammonium hydroxide, and 70 mL of 0.01 N $K_3Fe(CN)_6$ was added in 10-mL portions over 90 min. The solution was stirred for an additional 30 min. The pH was decreased to 5.0 with a few drops of glacial acetic acid, and the ferrocyanide and excess ferricyanide were removed by stirring the solution with 30 mL (settled volume) of anion-exchange resin Amberlite IRA-45 (Cl^- form). After the mixture was stirred for 30 min, the resin was filtered off and washed with 3 × 30 mL of 30% aqueous acetic acid. The solution was evaporated dow to 200 mL on a rotary evaporator in vacuo at 25 °C and lyophilized. The residue was dissolved in 10 mL of 15% acetic acid and centrifuged 10 min at 4000 rpm to precipitate a small amount of ferricyanide that had not been removed previously. The supernatant was applied to a Sephadex G-15 gel-filtration column (50 × 3.2 cm). The major peak was isolated and lyophilized. The resulting powder was dissolved in 2 mL of 20% acetonitrile in 0.1% aqueous trifluoroacetic acid (TFA) and purified on a Vydac 218TP1010 C_{18} RP-HPLC column (25 cm × 1 cm). HPLC conditions used to separate the D- and L-*erythro*-β-MePhe-containing peptide diastereoisomers were as follows: linear gradient elution starting with 20% CH_3CN in 0.1% aqueous TFA, 1%/min for 15 min at a flow rate of 4 mL/min, following by 5 min isocratic elution with 35% CH_3CN in 0.1% TFA. The more lipophilic impurities were eluted with 80% CH_3CN in 0.1% TFA for 5 min. The column was equilibrated for 10 min with 20% CH_3CN in 0.1% TFA before the next injection of peptide. Approximately 5 mg of crude peptide were purified per injection. Two major peaks corresponding to the two diastereomeric peptides were each isolated as a white powder. The yield of the first peak eluted was 30 mg, and the yield of the second peak was 35 mg.

The determination of the identity of the optically pure enantiomers was carried out by enzymatic methods. A sample of 1 mg of each peptide was hydrolyzed with 6 N HCl for 24 h at 110 °C. The HCl was removed in vacuo, and the residue was dissolved in 200 μL of water. A 100 μL of the solution was diluted with 10 μL of Tris buffer, pH = 7.2, then 5 mg (2.25 units) of L-amino acid oxidase (Sigma, St. Louis, MO) was added, and the mixture was incubated for 24 h at 37 °C. An additional 2.5 mg of enzyme was added, and the incubation was continued for another 24 h. The sample was diluted with 1 mL of citrate buffer, pH = 2.2 (Beckman, Palo Alto, CA). Amino acid analyses were performed on both the treated and non-enzyme-treated peptide samples. In the enzyme-treated sample of the first peptide diastereoisomer the amino acid peak corresponding to (2*S*,3*S*)-β-methylphenylalanine could not be detected by amino acid analysis. However, the corresponding peak was present in the nontreated sample. Since the L-amino acid oxidase enzyme decomposes (2*S*,3*S*)-β-MePhe, the first peptide eluted corresponds to the L isomer [(2*S*,3*S*)-β-MePhe⁴]DPDPE and the second peptide eluted is the D isomer [(2*R*,3*R*)-β-MePhe⁴]DPDPE. Similar results were obtained by chiral TLC.[35] Amino acid analysis for **2**: Gly 1.00 (1.00), Tyr 0.91 (1.00), (*S*,*S*)-β-MePhe 1.00 (1.00). For **3**: Gly 1.00 (1.00), Tyr 0.94 (1.00), (2*R*,3*R*)-β-MePhe 1.00 (1.00). See Table IV for analytical data for the purified products.

[D-Pen²,(2*S*,3*R*)-β-MePhe⁴,D-Pen⁵]enkephalin (4) and [D-Pen²,(2*R*,3*S*)-β-MePhe⁴,D-Pen⁵]enkephalin (5). The title compounds were prepared from 1 mmol of N^{α}-Boc-D-Pen(*S*-*p*-MeBzl)-O-resin as for **2** and **3** above except that N^{α}-Boc-*threo*-D,L-β-MePhe was added to the growing peptide chain instead of N^{α}-Boc-*erythro*-D,L-β-MePhe. This resulted in 3.19 g of N^{α}-Boc-Tyr-D-Pen(*S*-*p*-MeBzl)-Gly-*threo*-D,L-β-MePhe-D-Pen(*S*-*p*-MeBzl)-O-resin. The peptide resin was treated with HF as before, and the peptide was isolated, cyclized to the disulfide, and purified as for **2** and **3** above. Enzymatic methods again revealed that the first peptide eluted by HPLC corresponded to the (*S*,*R*)-L-β-MePhe-containing diastereoisomer **4**, and the second peptide eluted corresponded to the (*R*,*S*)-D-β-MePhe-containing diastereoisomer **5**. The synthesis yielded 15 mg of **4** and 20 mg of **5** as white powders. Similar results were obtained by chiral TLC.[35] Amino acid analysis for **4**: Gly 1.00 (1.00), Tyr 0.92 (1.00), (2*S*,3*R*)-β-MePhe 1.00 (1.00). For **5**: Gly 1.00 (1.00), Tyr 0.93 (1.00), (2*R*,3*S*)-β-MePhe 1.00 (1.00). See Table IV for analytical data for the purified products.

[D-Pen²,(2*S*,3*S*)-β-Me-*p*-NO₂Phe⁴,D-Pen⁵]enkephalin (6) and [D-Pen²,(2*R*,3*R*)-β-Me-*p*-NO₂Phe⁴,D-Pen⁵]enkephalin (7). The title compounds were prepared from 1.5 mmol of N^{α}-Boc-D-Pen(*S*-*p*-MeBzl)-O-resin as for **2** and **3** above except that racemic N^{α}-Boc-*erythro*-β-Me-*p*-NO_2Phe-OH was used in place of racemic β-MePhe in the synthesis of the peptide chain. This yielded 2.43 g of N^{α}-Boc-Tyr-D-Pen(*S*-*p*-MeBzl)-Gly-*erythro*-(D,L)-β-MePhe-D-Pen(*S*-*p*-MeBzl)-O-resin. The peptide resin was treated with HF as before, and the peptide was isolated and cyclized to the disulfide. The crude peptide mixture was purified by gel filtration on a Sephadex G-10 column with 15% acetic acid and then a part of it (100 mg) by HPLC as described previously to give 18 (**6**) and 23 mg (**7**) of the two peptide diastereomers. Enzymatic and chiral TLC methods again revealed that the first peptide eluted by HPLC corresponded to the (2*S*,3*S*)-β-Me-*p*-NO_2Phe⁴-containing diastereomer **6**, and the second peptide eluted corresponded to the (2*R*,3*R*)-β-Me-*p*-NO_2Phe⁴-containing diastereomer **7**. Amino acid analysis for **6**: Gly 1.00 (1.00), Tyr 0.89 (1.00), (*S*,*S*)-β-Me-*p*-NO_2Phe 1.00 (1.00). For **7**: Gly 1.00 (1.00), Tyr 0.90 (1.0), (*R*,*R*)-β-Me-*p*-NO_2Phe 1.02 (1.00). Analytical data for the products are given in Table IV.

[D-Pen²,(2*S*,3*R*)-β-Me-*p*-NO₂Phe⁴,D-Pen⁵]enkephalin (8) and [D-Pen²,(2*R*,3*S*)-β-Me-*p*-NO₂Phe⁴,D-Pen⁵]enkephalin (9). The title compounds were prepared from 1.25 mmol of N^{α}-Boc-D-Pen(*S*-*p*-MeBzl)-O-resin as for **2** and **3** above except that racemic N^{α}-Boc-*threo*-β-Me-*p*-NO_2Phe-OH was used in place of racemic *erythro*-β-MePhe in the synthesis of the peptide chain. This yielded N^{α}-Boc-Tyr-D-Pen(*S*-*p*-MeBzl)-Gly-*threo*-D,L-β-Me-*p*-NO_2-Phe-D-Pen(*S*-*p*-MeBzl)-O-resin. The peptide resin was treated with HF as before, and the peptide was isolated and cyclized to the disulfide. The crude peptide mixture was purified by gel filtration on a Sephadex G-10 column with 15% acetic acid and a part of it (100 mg) by HPLC as described previously to give 17 mg of **8** and 20 mg of **9**. Enzymatic and chiral TLC methods again revealed that the first peptide eluted by HPLC corresponded to the (2*S*,3*R*)-β-Me-*p*-NO_2Phe⁴-containing diastereomer **8**, and the second peptide eluted corresponded to the (2*R*,3*S*)-β-Me-*p*-NO_2Phe⁴-containing diastereomer **9**. Amino acid analysis for **8**: Gly 1.00 (1.00), Tyr 0.92 (1.00), (*S*,*R*)-β-Me-*p*-NO_2Phe 0.98 (1.00). For **9**: Gly 1.00 (1.00), Tyr 0.90 (1.00), (*R*,*S*)-β-Me-*p*-NO_2Phe 1.01 (1.00). Analytical data for the products are given in Table IV.

GPI and MVD Bioassays. Electrically induced smooth muscle contractions of mouse vas deferens and guinea pig ileum longitudinal muscle–myenteric plexus were used as a bioassay.[40] Tissues came from male ICR mice (weight 25–30 g) and from male Hartley guinea pigs (weight 150–400 g). The tissues were tied to gold chains with suture silk, suspended in 20-mL baths containing 37 °C oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate solution (magnesium-free for the MVD), and allowed to equilibrate for 15 min. The tissues were then stretched to optimal length previously determined to be 1 g of tension (0.5 g for MVD) and allowed to equilibrate for 15 min. The tissues were stimulated transmurally between platinum plate electrodes at 0.1-Hz 0.4-ms pulses (2.0-ms pulses for MVD), and supramaximal voltage. Drugs were added to the baths in 20–60-μL volumes. The agonists remained in contact with the tissue for 3 min and the baths were then rinsed several times with fresh Krebs solution. Tissues were given 8 min to reequilibrate and regain predrug contraction height. Percent inhibition was calculated by using the average contraction

(40) Shook, J. E.; Pelton, J. T.; Wire, W. S.; Herning, L. D.; Hruby, V. J.; Burks, J. F. *J. Pharmacol. Exp. Ther.* **1987**, *240*, 772–777.

height for 1 min preceding the addition of the agonist divided by the contraction height 3 min after exposure to the agonist. IC_{50} estimates and relative potency estimates were determined by fitting the mean data to logistic equation by using a computerized nonlinear least-squares method.[41] The number of replications done was two to four experiments for each compound in duplicate. In some cases, the weak μ agonist actions of these analogues did not permit completion of dose–response curves in the GPI.

Radioreceptor Assay. Adult male Sprague–Dawley rats (200–250 g) were sacrificed and brains immediately removed and placed on ice. Whole brain minus cerebellum was homogenized with a Polytron homogenizer (Brinkman, setting 5, 15 s). The homogenate was preincubated at 25 °C for 30 min three times (three washes with 20 volumes of fresh 50 mM Tris buffer) to remove endogenous opioids and then centrifuged two times at 43000*g* for 10 min before use in the radioreceptor assay.

Binding affinities vs [^{3}H]DPDPE, [^{3}H][*p*-ClPhe^4]DPDPE, and [^{3}H]CTOP (all from New England Nuclear, Boston, MA) were measured by rapid filtration methods as described for [*p*-ClPhe^4]DPDPE[26] and CTOP.[27] A 100-μL aliquot of rat brain homogenate (0.5% final) was incubated with either 1.0 nM [^{3}H]DPDPE or 0.8 nM [^{3}H][*p*-ClPhe^4]DPDPE or 0.5 nM [^{3}H]-CTOP in a total volume of 1 mL of 50 mM Tris-HCl, pH 7.4, at ~5 °C containing 5 mM $MgCl_2$, bovine serum albumin (1 mg/mL), and phenylmethanesulfonyl fluoride (100 μL). Steady-state binding experiments were carried out at 25 °C for 120 min. All binding measurements were done in duplicate and two to four duplicate experiments were done for each compound. The radioligand displaced by 1 μM naltrexone hydrochloride was defined as specific tissue binding. The binding reaction was terminated by rapid filtration of samples through GF/B Whatman glass-fiber filter strips pretreated with 0.1% polyethylenimine solution with a Brandel cell harvester; this was followed immediately by three rapid washes with 4-mL aliquots of ice-cold saline solution. Filters were removed and allowed to dry before assaying filter-bound radioactivity by liquid scintillation spectrophotometry (45% efficiency). Binding data were analyzed by nonlinear regression methods.[26,27] IC_{50} affinity constants were converted to K_i values by the Cheng and Prusoff[42] equation using K_D values for DPDPE, [*p*-ClPhe^4]DPDPE, and CTOP of 4.05, 0.328, and 0.160, respectively.

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Registry No. 1, 88373-73-3; **2**, 133098-42-7; **3**, 133098-43-8; 4, 133098-44-9; **5**, 133098-45-0; **6**, 129073-55-8; **7**, 129063-25-8; **8**, 129073-56-9; **9**, 129048-79-9; DL-*erythro*-β-methyl-Phe, 25488-24-8; DL-*threo*-β-methyl-Phe, 80372-54-9; L-*erythro*-β-methyl-Phe, 25488-25-9; D-*erythro*-β-methyl-Phe, 25488-26-0; DL-*erythro*-β-methyl-*p*-nitro-Phe, 133009-51-5; DL-*threo*-β-methyl-*p*-nitro-Phe, 133009-52-6; L-*erythro*-β-methyl-*p*-nitro-Phe, 128502-51-2; D-*erythro*-β-methyl-*p*-nitro-Phe, 128502-68-1; BOC-DL-*erythro*-β-methyl-Phe, 115132-19-9; BOC-DL-*threo*-β-methyl-Phe, 133009-53-7; BOC-DL-*erythro*-β-methyl-*p*-nitro-Phe, 133009-54-8; BOC-DL-*threo*-β-methyl-*p*-nitro-Phe, 133009-55-9.

(41) Statistical Consultants, Inc., Am. Stat. 1986, *40*, 52.

(42) Cheng, Y.-C.; Prusoff, W. H. *Biochem. Pharmacol.* 1973, *22*, 3099–3102.

Lipophilic Glycosyl Phosphotriester Derivatives of AZT: Synthesis, NMR Transmembrane Transport Study, and Antiviral Activity

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Phosphate derivatives of AZT esterified with a carbohydrate (D-glucose, D-mannose, and ethyl D-mannopyranoside) and a hexadecyl chain were prepared from glucose 6-phosphate and D-mannose precursors. The ^{31}P NMR study of the mannosyl phosphotriester series in the presence of large unilamellar vesicles demonstrated either an interaction with the external lipid layer or a transmembrane transport into the intravesicular interface. The antiviral activity, measured by the inhibition of cytopathogenicity on different infected cells and of reverse transcriptase activity in the supernatant of cultures, appeared to be comparable to that of AZT, in the micromolar range.

Among the numerous approaches for the control of acquired immunodeficiency syndrome (AIDS) caused by the retrovirus HIV, so far only 3′-azido-3′-deoxythymidine, AZT,[1] has been identified as a clinically useful drug and approved specifically for treatment.[2–4] AZT acts as an inhibitor of the viral reverse transcriptase after its conversion into 5′-triphosphate by host cell thymidine and thymidilate kinases.[5] In contrast with other nucleoside transport, AZT permeates the membrane by passive diffusion,[6] crosses over the blood–brain barriers,[3–7] and partially reverses the neurological dysfunction due to HIV in some patients.[8] However the therapeutic potential of AZT is limited by serious adverse reactions, particularly bone marrow suppression,[9] and intensive efforts are still needed

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(1) Horwitz, J. P.; Chua, J.; Noel, M. *J. Org. Chem.* 1964, *29*, 2076.
(2) Mitsuya, H.; Weinhold, K. J.; Furman, P. A.; St. Clair, M. H.; Nusinoff-Lehrman, S.; Gallo, R. C.; Bolognesi, D.; Barry, D. W.; Broder, S. *Proc. Natl. Acad. Sci. U.S.A.* 1985, *82*, 7096.
(3) Yarchoan, R.; Klecker, R. W.; Weinhold, K. J.; Markham, P. D.; Lylerly, H. K.; Durack, D. T.; Gelmann, E.; Nusinoff-Lehrman, S.; Blum, R. M.; Barry, D. W.; Shearer, G. M.; Fischl, M. A.; Mitsuya, H.; Gallo, R. C.; Collins, J. M.; Bolognesi, D. P.; Myers, C. E.; Broder, S. *Lancet* 1986, *i*, 575.
(4) Fischl, M. A.; Richman, D. D.; Grieco, M. H.; Gottlieb, M. S.; Volberding, P. A.; Laskin, O. L.; Leedom, J. M.; Groopman, J. E.; Mildvan, D.; Schooley, R. T.; Jackson, G. G.; Durack, D. T.; Phil, D.; King, D.; The AZT Collaborative Working Group *N. Engl. J. Med.* 1987, *317*, 185.
(5) Mitsuya, H.; Broder, S. *Nature (London)* 1987, *325*, 773.
(6) Zimmerman, T. P.; Mahony, W. B.; Prus, K. L. *J. Biol. Chem.* 1987, *262*, 5748.

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