(m, 2 H), 3.79 (s, 3 H), 4.05-4.07 (t, J = 6.5 Hz, 2 H), 4.97-5.02 (d, 1 H), 7.37-7.43 (m, 2 H), 7.56-7.57 (m, 3 H), 7.68-7.71 (d, J = 9 Hz, 1 H), 8.01-8.04 (m, 2 H), 10.01 (s, 1 H); IR (KBr) 3400, 2940, 1620 cm^{-1}. Anal. Calcd for $C_{27}H_{33}NO_5$·HCl·0.5H_2O: C, 65.25; H, 7.09; N, 2.81. Found: C, 65.20; H, 7.02; N, 2.81.

Method J. Illustrated by the Synthesis of 6-[(6-Aminohexyl)oxy]flavone (80). A mixture of 6-hydroxyflavone (5.00 g, 21 mmol), 6-bromocapronitrile (7.7 g, 44 mmol), and potassium carbonate (12 g, 88 mmol) in 200 mL of acetone was refluxed for 16 h. The reaction was cooled to room temperature and filtered. The mother liquors were concentrated in vacuo to give an off-white solid which was triturated with 25 mL of ether and filtered to give 6.32 g (90%) of 6-[(5-cyanopentyl)oxy]flavone (91), which was pure by ^{1}H NMR: ($CDCl_3$) δ 1.70 (m, 2 H), 1.75 (m, 2 H), 1.88 (m, 2 H), 2.41 (t, J = 6.7 Hz, 2 H), 4.09 (t, J = 6.2 Hz, 2 H), 6.82 (s, 1 H), 7.30 (m, 1 H), 7.54 (m, 5 H), 7.92 (m, 2 H).

6-[(5-Cyanopentyl)oxy]flavone (1.73 g, 4.4 mmol) and cobalt(II) chloride hexahydrate (2.09 g, 8.8 mmol) were dissolved in 50 mL of EtOH. Sodium borohydride (0.84 g, 22 mmol) was added in portions over 5 min at room temperature. The addition was accompanied by the evolution of gas and the solution turned black. After stirring at room temperature for 1 h, TLC (silica gel; ether; UV visualization) indicated complete reaction. The reaction was poured into 100 mL of 3 N HCl and the resulting solution was stirred at room temperature for 1.5 h. The solution was filtered and concentrated in vacuo to ca. 100 mL. Then ammonium hydroxide was added to adjust the pH to 8. A yellow precipitate was collected by filtration and dried to give 1.03 g (2.6 mmol) of the amine. The amine was dissolved in 300 mL of EtOH and 2.6 mL of a 1 M anhydrous solution of HCl in ether was added. The solution was concentrated in vacuo to ca. 150 mL and cooled in the freezer. The resulting orange precipitate was collected by vacuum filtration to give 0.72 g (44%) of 6-[(6-aminohexyl)-oxy]flavone hydrochloride (80): mp 218-221 °C dec; ^{1}H NMR (DMSO-d_6) δ 1.43 (m, 4 H), 1.58 (m, 2 H), 1.76 (m, 2 H), 2.77 (m, 2 H), 4.08 (t, J = 6.4 Hz, 2 H), 7.00 (s, 1 H), 7.43 (m, 2 H), 7.60 (m, 3 H), 7.75 (m, 1 H), 7.8 (br, 2 H), 8.09 (m, 2 H); IR (KBr) 3428, 2934, 2875, 1630 cm^{-1}. Anal. Calcd for $C_{21}H_{23}NO_3$·HCl: C, 67.46; H, 6.47; N, 3.75; Cl, 9.48. Found: C, 67.09; H, 6.67; N, 3.50; Cl, 8.89.

Substitution on the Phe^3 Aromatic Ring in Cyclic δ Opioid Receptor-Selective Dermorphin/Deltorphin Tetrapeptide Analogues: Electronic and Lipophilic Requirements for Receptor Affinity†

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In an effort to explore structural features affecting receptor recognition in a series of conformationally restricted tetrapeptides related to the cyclic, δ opioid receptor-selective analogue, Tyr-D-Cys-Phe-D-PenOH, electronic, lipophilic, and steric effects at the Phe^3 residue were assessed by substitution at different positions of the side-chain aromatic ring by halogens, alkyl, hydroxyl, and nitro groups. Effects on opioid receptor binding affinity and selectivity were determined. The results, which are generally consistent with reports of analogous modifications in linear and cyclic pentapeptide enkephalins, indicate that steric, lipophilic, and electronic properties are all important determinants of δ opioid receptor recognition. Specifically, modifications which increase lipophilicity or exert electron-withdrawing effects on the aromatic ring enhance binding affinity, while hydrophilic, bulky, or electron-releasing modifications are detrimental. These observations are in excellent agreement with quantitative structure-activity relationship (QSAR) results reported for Phe^4 modifications in linear opioid pentapeptide enkephalin analogues, suggesting that the Phe^3 tetrapeptide side chain and the Phe^4 pentapeptide side chain interact with the same δ receptor binding subsite.

Introduction

While convincing in vivo and in vitro pharmacological evidence of opioid receptor heterogeneity has long been available[1-4] and the existence of at least μ, δ, and κ classes of opioid receptors is widely accepted, the elucidation of the specific structural and conformational requirements for ligand interaction with these different receptor types remains elusive. Since it is conceivable that the μ, δ, and κ receptor types may mediate different pharmacological events, such knowledge may lead to the eventual design of selective enkephalin analogues or other analgesic compounds which exhibit desired pharmacological actions, yet are devoid of negative side effects. In our efforts to uncover both the structural and conformational features required of the ligand for δ and μ receptor recognition and to develop potent ligands with high selectivity for a single receptor type, we have employed the approach of designing enkephalin analogues into which conformational restrictions have been incorporated. Since this reduces the number of spatial orientations a large molecule may assume, peptide analogues designed with the appropriate conformational constraints may display selectivity resulting from the ability to adopt the required binding conforma-

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† Abbreviations recommended by IUPAC-IUB Commission of Biochemical Nomenclature have been used. Other abbreviations: ACN, acetonitrile; N^α-Boc, N^α-*tert*-butyloxycarbonyl; COSY, correlation spectroscopy; DAMGO, Tyr-D-Ala-Gly-*N*-MePhe-Gly-ol; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DPDPE, Tyr-D-Pen-Gly-Phe-D-PenOH; DPM, disintegrations per minute; FAB-MS, fast atom bombardment mass spectrometry; HOAc, acetic acid; HOBt, 1-hydroxybenzotriazole; metkephamide, Tyr-D-Ala-Gly-Phe-*N*-MeMetNH$_2$; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; RP-HPLC, reverse-phase high performance liquid chromatography; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TSP, 3-(trimethylsilyl)-propionic acid; Tris, tris(hydroxymethyl)aminomethane; U69,593, 5α,7α,8β-(-)-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide.

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Table I. Opioid Receptor Binding Profiles of Cyclized Tetrapeptides

peptide analogue	compd	binding: K_i (nM) DAMGO[a]	DPDPE[b]	U69,593	$K_i(\mu)/K_i(\delta)$
Tyr-D-Pen-Gly-Phe-D-PenOH	(DPDPE)	810 ± 66	3.98 ± 0.46	>10000	204
Tyr-D-Cys-Phe-D-PenOH (JOM-13)	1	107 ± 1.0	1.79 ± 0.11	>10000	60
Tyr-D-Cys-*p*-FPhe-D-PenOH	2	161 ± 11	1.10 ± 0.08	>10000	146
Tyr-D-Cys-*o*-FPhe-D-PenOH	3	91.9 ± 7.2	3.27 ± 0.34	>10000	28.1
Tyr-D-Cys-*m*-FPhe-D-PenOH	4	55.5 ± 5.8	2.89 ± 0.35	>10000	19.2
Tyr-D-Cys-*p*-ClPhe-D-PenOH	5	327 ± 23	0.98 ± 0.30	~1000	334
Tyr-D-Cys-*p*-MePhe-D-PenOH	6	1750 ± 420	5.38 ± 0.52	>10000	325
Tyr-D-Cys-*p*-*tert*-BuPhe-D-PenOH	7a	~10000	648. ± 80.	>10000	15
Tyr-D-Cys-*p*-*tert*-BuPhe-D-PenOH	7b	~10000	36.7 ± 2.0	>10000	270
Tyr-D-Cys-Tyr-D-PenOH	8	3450 ± 120	25.6 ± 1.8	>10000	135
Tyr-D-Cys-*m*-Tyr-D-PenOH	9a	3400 ± 2300	148. ± 7.0	>10000	22.3
Tyr-D-Cys-*m*-Tyr-D-PenOH	9b	712 ± 130	9.29 ± 0.81	>10000	76.6
Tyr-D-Cys-*p*-NO_2Phe-D-PenOH	10	137 ± 8.0	1.66 ± 0.13	>10000	82.5

[a] DAMGO = [^{3}H][D-Ala^2, *N*-MePhe^4, Gly^5-ol]enkephalin. [b] DPDPE = [^{3}H][D-Pen^2, D-Pen^5]enkephalin.

tion for only one receptor type.

Using this approach, we have previously developed a series of enkephalin analogues in which conformational restriction via cyclization of side-chain sulfurs led to high δ receptor selectivity.[5-7] The most selective analogue in this series, Tyr-D-Pen-Gly-Phe-D-PenOH (DPDPE), where Pen = penicillamine = β,β-dimethylcysteine, is widely employed as a standard of δ receptor selectivity. More recently, we have described a potent, δ opioid receptor-selective tetrapeptide, Tyr-D-Cys-Phe-D-PenOH (JOM-13, 1), also cyclized via a disulfide bond involving the Cys and Pen side-chain sulfur atoms.[8] This tetrapeptide is closely related in structure to the DPDPE series of enkephalin pentapeptides, with the exception that it lacks the central Gly^3 residue. The peptides in this series therefore more closely resemble tetrapeptide analogues of the frog-skin opioid heptapeptides dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-SerNH_2)[9] and the deltorphins (Tyr-D-Ala-Phe-X-Val-Val-GlyNH_2; X = Asp, Glu).[10] JOM-13, itself, actually exhibits a higher affinity for the δ opioid receptor than does DPDPE, with only a minor sacrifice of selectivity.[8] Since JOM-13 and analogues related to it lack the relatively flexible central glycine residue found in DPDPE, they exhibit less conformational lability than DPDPE and thus lend themselves more readily to conformational analysis. This tetrapeptide series, then, represents a valuable tool for mapping the binding site of the δ opioid receptor and for assessing the disparities between δ and μ receptors, as well as for the development of more potent and selective opioid peptides.

Recognizing that structure–conformation relations pertaining to the linear and cyclic pentapeptide enkephalins may not apply to the cyclic tetrapeptide series and that the tetrapeptides might interact differently with δ opioid receptors than do the pentapeptides, we have synthesized several analogues of JOM-13 with modifications designed to explore the effect of substitution of amino acids with varying steric, electronic, and lipophilic properties at each residue. We have focussed chiefly on the aromatic residues Tyr and Phe, which are generally accepted as being of major importance in opioid receptor recognition. In the present report we describe the effect of modifications to the Phe^3 aromatic side chain which alter the electronic, steric, and lipophilic–hydrophilic characteristics of the residue 3 side chain. In general, the effects of such modifications mirror those observed in linear and cyclic enkephalin pentapeptides and indicate, consistent with our earlier suggestion,[11,12] that these tetrapeptides and pentapeptides interact similarly with the δ opioid receptor.

Results and Discussion

The effects of modifying the electronic, lipophilic, and steric characteristics of the third residue side-chain function of the lead tetrapeptide, Tyr-D-Cys-Phe-D-PenOH (1) were assessed by incorporation of *p*-FPhe (2), *o*-FPhe (3), *m*-FPhe (4), *p*-ClPhe (5), *p*-MePhe (6), *p*-*t*-BuPhe (7a and 7b), Tyr (8), *m*-Tyr (9a and 9b), and *p*-

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Table II. Physicochemical Data for Cyclized Peptides

peptide analogue	compd	elution time (min) 10–70/30[a]	elution time (min) 0–70/70[b]	purity (%)[c]	[O] yield (%)[d]	TLC R_f[e] A	TLC R_f[e] B	TLC R_f[e] C	FAB-MS mw
Tyr-D-Cys-*p*-FPhe-D-PenOH	**2**	17	35	100	18	0.73	0.70	0.70	578
Tyr-D-Cys-*o*-FPhe-D-PenOH	**3**	17	30	99	46	0.68	0.54	0.77	578
Tyr-D-Cys-*m*-FPhe-D-PenOH	**4**	16	32	99	23	0.69	0.54	0.70	578
Tyr-D-Cys-*p*-ClPhe-D-PenOH	**5**	17	34	100	47	0.69	0.60	0.63	594
Tyr-D-Cys-*p*-MePhe-D-PenOH	**6**	16	32	100	23	0.66	0.55	0.62	574
Tyr-D-Cys-*p*-tBuPhe-D-PenOH	**7a**	22	44	96	28	0.55	0.43	0.61	616
Tyr-D-Cys-*p*-tBuPhe-D-PenOH	**7b**	20	40	99	18	0.64	0.62	0.65	616
Tyr-D-Cys-Tyr-D-PenOH	**8**	13	27	97	24	0.59	0.55	0.64	576
Tyr-D-Cys-*m*-Tyr-D-PenOH	**9a**	15	30	95	>14	0.49	0.35	0.57	576
Tyr-D-Cys-*m*-Tyr-D-PenOH	**9b**	14	29	98	>9	0.57	0.51	0.62	576
Tyr-D-Cys-*p*-NO_2Phe-D-PenOH	**10**	17	33	98	56	0.65	0.54	0.64	605

[a] HPLC elution time using a linear gradient of 10–70% organic component in 30 min at a flow rate of 1 mL/min. Solvent system was 0.1% (w/v) TFA in water/0.1% (w/v) TFA in acetonitrile. The solvent front eluted at 3.0 min. [b] HPLC elution time using a linear gradient of 0–70% organic component in 70 min at a flow rate of 1 mL/min. Solvent system was 0.1% (w/v) TFA in water/0.1% (w/v) TFA in acetonitrile. The solvent front eluted at 3.0 min. [c] Purity of final cyclized peptide as assessed by HPLC peak integration from chromatograms at 230 nm. [d] Yield in peptide cyclization/oxidation reaction, after purification. [e] R_f values for thin-layer chromatograms in solvent systems: (A) 1-butanol/acetic acid/water (4:1:5, organic component only); (B) 1-butanol/water (containing 3.5% acetic acid and 1.5% pyridine) (1:1, organic component only); (C) *n*-amyl alcohol/pyridine/water (7:7:6).

NO_2Phe (**10**) as residue 3 replacements for Phe. Opioid receptor binding affinities of these new analogues for μ, δ, and κ receptor types, reported as K_i values (± standard errors of the mean) against [^{3}H]DAMGO (μ), [^{3}H]DPDPE (δ), and [^{3}H]U69,593 (κ), can be found in Table I, along with corresponding data for DPDPE and **1**. The ratio of the μ and δ K_i values provides a measure of the δ vs μ selectivity of the compound. Like those peptides in the DPDPE series, none of these analogues displays high affinity for the κ receptor. In all but one case, κ receptor binding affinity was very weak (K_i > 10 000 nM). For the sole exception, analogue **5**, κ affinity, calculated to be ~ 1000 nM, was 1000-fold weaker than δ and 3-fold weaker than μ affinity. Accordingly, κ binding behavior of these peptides is considered no further here. Physicochemical data for all newly reported peptides are presented in Table II.

Analogues **2–5**, all of which contain halogenated phenylalanine in residue 3, probe the effects of varying the aromatic ring position of the halogen substitution (**2–4**) and of altering the size and electronic character of the halogen (**2** and **5**). For all four analogues, the halogen substitution is expected to increase lipophilicity and exert an electron-withdrawing effect relative to the unsubstituted phenylalanine side chain. As can be seen in Table I, altering the aromatic ring position of fluorine substitution in analogues **2–4** significantly affects both binding affinity and selectivity. While para substitution results in the lowest μ receptor affinity (vs ortho and meta), it yields the highest δ binding affinity. Accordingly, **2** exhibits considerably higher δ binding selectivity than **3** and **4**. The trend in δ binding is particularly interesting. The observation that δ binding affinity decreases as follows: *p*-FPhe > *m*-FPhe > *o*-FPhe, is consistent both in rank order and in magnitude of effect with observations reported for similar substitutions in linear opioid pentapeptides related to metkephamide.[13] Several possible explanations for the observed trend can be considered. Since the *o*-F, *m*-F, and *p*-F modifications vary in steric characteristics, steric effects on the peptide ligand conformation or effects on ligand–receptor recognition may underlie the observed affinity differences. However, these steric differences should be minimal due to the small van der Waals radius of fluorine. Indeed, we have recently reported that substitution of Phe^3 in **1** by 3-(1-naphthyl)alanine and by 3-(2-naphthyl)alanine, substitutions with more significant steric effects than those considered here, do not diminish δ affinity.[12] Thus, differential steric effects seem an unlikely explanation of the observed variation in binding affinity. A more likely explanation is suggested by the data compiled in Table II, which indicate that lipophilicity, as measured by RP-HPLC retention times, in this structurally similar subseries of three compounds, is proportional to δ binding affinity. While such a correlation need not imply causality, the interpretation that, other factors being equal, increased lipophilicity of the residue 3 side chain results in enhanced binding affinity seems a reasonable one. An alternate, indirectly related hypothesis also warrants consideration. The variation in lipophilicity with position of the electronegative fluorine substituent on the aromatic ring most likely can be attributed to differences in the magnitude and direction of the dipole moment of the side chain. It is thus possible that the nature of dipole moment of the residue 3 side chain influences interaction with the receptor and that the variation in δ (and perhaps μ) binding affinity observed in analogues **2–4** may reflect this.

Compound **5**, the *p*-ClPhe^3-substituted analogue, displays enhanced δ affinity and diminished μ affinity relative to **1** and consequently shows significantly enhanced δ receptor selectivity. Compared to **2**, analogue **5** exhibits similar δ receptor binding affinity and 2-fold reduced μ affinity. These results are in excellent agreement with observations reported for similar modifications in DPDPE[14] and in linear enkephalins.[13] The high δ selectivity of **5**, ca. 5-fold that of the lead compound **1** and >50% higher than that of DPDPE, coupled with the en-

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hanced δ receptor affinity of **5**, make it an attractive candidate for further study. This is also suggested by preliminary results from the mouse vas deferens (MVD) bioassay, in which activity primarily at δ receptors is assessed. We have observed (F. Porreca, personal communication) that **5** has very high potency (IC_{50} = 1.5 nM) in this assay and is in fact about 3-fold more potent than **1**.[11] This is similar to results obtained for **2**,[11] in which a slightly greater enhancement of bioassay potency vs binding affinity is also observed and is consistent with results reported for *p*-ClPhe[4]- and *p*-FPhe[4]-substituted analogues of DPDPE.[14]

The *p*-methyl and *p*-*tert*-butyl substitutions on the phenylalanine aromatic ring in compounds **6**, **7a**, and **7b**, like the halogen substitutions in **2–5**, increase lipophilicity; however, the alkyl substitutions are electron-releasing rather than electron-withdrawing. Consequently, the relative importance of electronic and lipophilic effects of substituents at this residue can be assessed. On the basis of RP-HPLC retention times tabulated in Table II, the *p*-methyl substituent in **6** results in increased lipophilicity similar to that observed for the *p*-FPhe[3] analogue **2**. Comparison of van der Waals radii indicates similar steric characteristics for **6** and the *p*-ClPhe analogue **5**. As shown in Table I, the δ receptor binding affinity of **6** is reduced ca. 5-fold relative to **2** and **5**, a result which can therefore be attributed to the difference in electronic properties associated with the modifications. A similar reduction in μ receptor binding affinity of **6** vs **2** and **5** is also observed. As a result, **6** displays excellent δ receptor selectivity, equivalent to that of **5**. The reduced binding affinity of **6** in this series is in good agreement with results reported by Hruby and co-workers for [*p*-MePhe[4]]DPDPE which indicated an approximate 6-fold reduction in binding affinity compared with DPDPE.[15] Analogues **7a** and **7b**, which contain *p*-*tert*-butylphenylalanine in residue **3**, exhibit further reductions in binding affinity. The considerably reduced δ affinity of **7a** compared with **7b** suggests that the former contains D-*p*-*t*-BuPhe and the latter, L-*p*-*t*-BuPhe, consistent with previous observations of large reductions in binding affinity in cyclic tetrapeptide and pentapeptide opioid analogues containing a D-amino acid in residue 3 or 4, respectively.[11,16] Given this tentative assignment, the ca. 7-fold reduction in δ binding affinity of **7b** vs **6** is most likely attributable to the increased bulk of the *tert*-butyl substitution, although the possible deleterious effects of increased lipophilicity and/or more pronounced electron-releasing tendency cannot be immediately excluded.

In the Tyr[3] analogue **8**, the *p*-hydroxyl substituent on residue 3, like the alkyl substituents in **6–7b**, is electron-releasing. In addition, it is similar in size to the *p*-methyl and *p*-chloro modifications in **6** and **5**, respectively. In contrast to the lipophilic characteristics of the halogens and the alkyl substituents, however, the hydroxyl moiety is hydrophilic (as is reflected by the earlier RP-HPLC elution time of **8**, as shown in Table II). Binding data presented in Table I indicate that **8** exhibits approximately 5-fold lower affinity for the δ receptor than does **6**. In view of the similar inductive and steric properties of the *p*-Me and *p*-OH substitutions, this reduced affinity can be attributed to the increased hydrophilicity accompanying the latter modification. The results obtained with the *m*-Tyr[3] analogues, **9a** and **9b**, are consistent with the observations made above. Like **7a** and **7b**, **9a** and **9b** were synthesized using a racemic mixture of the residue 3 amino acid, in this case D,L-*m*-Tyr. Relying on similar arguments to those used in the tentative assignment of **7a** and **7b**, we note that the greatly reduced binding affinity of **9a** suggests that this analogue contains D-*m*-Tyr in residue 3 and thus **9b** contained L-*m*-Tyr[3]. Comparison of binding data for **8** and **9b** than affords examination of the effect of location of the hydroxyl moiety on the aromatic ring. As seen from Table I, the *m*-Tyr[3] analogue **9b** displays approximately 3-fold higher δ (and 5-fold higher μ) binding affinity than does the Tyr[3] analogue **8**. The enhanced δ binding affinity displayed by **9b** vs **8**, which is opposite of the results observed in the fluorinated analogues **2** and **4**, apparently derives from the somewhat increased lipophilicity of **9b** relative to **8** (see RP-HPLC retention times in Table II) and from the altered electronic properties accompanying meta substitution of the hydroxyl which has a slight electron-withdrawing effect.

The results obtained with the *p*-NO_2Phe[3] analogue **10** are in accord with the observations made above which suggest that lipophilicity, and electronic and steric factors impact upon δ receptor binding affinity. The *p*-NO_2 substituent on the residue 3 aromatic side chain of analogue **10** is both electron withdrawing and lipophilic (see Table II), and on this basis would be expected to enhance binding affinity. However, **10** is virtually indistinguishable from the lead compound **1** in both δ and μ binding affinity, a likely consequence of the considerable increase in molecular volume accompanying the *p*-NO_2 substitution. The results obtained here are in agreement with previously reported effects of *p*-NO_2Phe[4] modification on δ and μ binding affinity in linear[13] enkephalin pentapeptides and on δ binding in the δ-selective cyclic DPDPE series.[15] In contrast to our findings, substitution of Phe[3] by *p*-NO_2Phe[3] in both linear[17] and cyclic[18] μ-selective dermorphin-related tetrapeptides induces a sharp decline in binding affinity at the μ receptor. To account for this differential effect of *p*-NO_2Phe[3] substitution in tetrapeptide opioids and *p*-NO_2Phe[4] substitution in opioid pentapeptides, Schiller proposed that the Phe[3] and Phe[4] side-chain phenyl rings do not interact with the same locus of the μ receptor.[17] Clearly, no such difference in binding modes for δ receptor binding of the tetrapeptide series reported here and previously examined pentapeptides is suggested by the data presented here, consistent with our earlier findings.[11,12]

Taken together, the data for the group of modifications incorporated into **2–10** indicate that lipophilic, electronic, and steric properties of residue 3 all play a role in influencing binding interactions. In general, small, lipophilic, electron-withdrawing substituents lead to enhanced δ receptor binding affinity, while electron-releasing or hydrophilic substituents, or those with large van der Waals radii which can interfere with receptor interaction, influ-

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ence binding deleteriously. These results are consistent with those reported by Fauchere in a QSAR analysis for pentapeptide analogues; in a study of Phe[4] substitutions in a series of linear enkephalin analogues, potency in the mouse vas deferens (MVD) bioassay (a prototypical δ receptor bioassay[4]) was proportional to lipophilicity (substituent hydrophobicity constant, π) and electron-withdrawing capacity (electronic substituent parameter, S) and inversely proportional to size.[19] This congruence between the effects of Phe[4] substitutions in pentapeptide opioids and Phe[3] substitutions in the tetrapeptide series reported here lends further support to our suggestion that these pentapeptides and tetrapeptides interact with the same δ receptor binding site.

Experimental Section

General Methods for Peptide Synthesis. Protected and unprotected amino acids, as well as coupling agents, were purchased from the following commercial sources: Aldrich Chemical Co., Pierce, Bachem Bioscience, Chemical Dynamics Corp., Sigma Chemical Co., Peptides International, Vega Biotechnologies, and Advanced ChemTech. N^{α}-(*tert*-Butyloxycarbonyl)-4′-*tert*-butyl-D,L-phenylalanine was generously provided by Dr. Don Hansen. Radioligands were purchased fron New England Nuclear and Amersham. Peptides were synthesized by standard solid phase procedures as previously described for the lead tetrapeptide 1,[8] using chloromethylated polystyrene (Merrifield) resin crosslinked with 1% divinylbenzene. Trifluoroacetic acid (TFA) was employed for deprotection, and dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) were used as coupling agents. α-Amino functions were protected with the *tert*-butyloxycarbonyl (Boc) group, and *p*-methylbenzyl protection was employed for the labile side-chain sulfhydryl groups of Cys and Pen. Deprotection and cleavage from the resin were accomplished by treatment with anhydrous hydrogen fluoride in the presence of 5% cresol and 5% *p*-thiocresol,[20] with stirring for 45 min at 0 °C. HF was subsequently removed by vacuum. Following extraction with 9:1 DMF/80% HOAc and dilution with 0.1% TFA, the resulting linear, free sulfhydryl-containing peptides were purified by reverse-phase high performance liquid chromatography (RP-HPLC) on a Vydac 218TP C-18 column (2.5 × 22 cm) using the solvent system 0.1% TFA in water/0.1% TFA in acetonitrile, by a gradient of 10–50% organic component in 40 min. Following lyophilization, treatment of an aqueous solution (pH 8.5) of the linear free sulfhydryl-containing compounds with $K_3Fe(CN)_6$ for ca. 1 h effected cyclization to disulfide analogues. Unless otherwise noted, the product cyclic peptides were then purified by RP-HPLC as described above, and pure fractions were pooled and lyophilized. Analytical data for the purified, cyclic peptides are summarized in Table II.

Tyr-D-Cys-*p*-FPhe-D-PenOH (2). The title compound was prepared by using 1 g of *S*-*p*-MeBzl-N^{α}-Boc-D-Pen Merrifield resin (substitution = 1.34 mmol/g). Successive couplings employed N^{α}-Boc-*p*-fluoro-L-phenylalanine, *S*-*p*-MeBzl-N^{α}-Boc-D-Cys, and N^{α}-Boc-L-Tyr. A 0.8-g sample of the protected, resin-bound precursor peptide was treated with 8 mL of HF, 0.4 g of *p*-cresol, and 0.4 g of *p*-thiocresol. After extraction from the resin with 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptide was subjected to semipreparative HPLC, which yielded, after lyophilization, 86.1 mg of pure linear peptide. A sample of 29.1 mg of this peptide was subjected to oxidation as described above, yielding after RP-HPLC, 5.1 mg of disulfide-containing peptide.

Tyr-D-Cys-*o*-FPhe-D-PenOH (3). The title compound was prepared similarly to 2, by using 1 g of *S*-*p*-MeBzl-N^{α}-Boc-D-Pen Merrifield resin (substitution = 0.94 mmol/g) and N^{α}-Boc-*o*-fluoro-L-phenylalanine in the first coupling. A 0.9-g sample of the protected, resin-bound precursor peptide was treated with 8 mL of HF, 0.4 g of *p*-cresol, and 0.4 g of *p*-thiocresol. After extraction from the resin with 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptide was subjected to semipreparative HPLC, which yielded, after lyophilization, 127 mg of pure linear peptide. A sample of 42.3 mg of this peptide was subjected to oxidation as described above, yielding after RP-HPLC, 19.3 mg of cyclized peptide.

Tyr-D-Cys-*m*-FPhe-D-PenOH (4). The title compound was prepared similarly to 2, by using N^{α}-Boc-*m*-fluoro-L-phenylalanine. A 0.9-g sample of the protected, resin-bound precursor peptide was treated with 8 mL of HF, 0.4 g of *p*-cresol, and 0.4 g of *p*-thiocresol. After extraction into 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptide was subjected to semipreparative HPLC, which yielded, after lyophilization, 117 mg of pure linear peptide. A sample of 37.5 mg of this peptide was subjected to oxidation as described above, yielding after RP-HPLC, 8.7 mg of disulfide-containing peptide.

Tyr-D-Cys-*p*-ClPhe-D-PenOH (5). The title compound was prepared by using 1 g of *S*-*p*-MeBzl-N^{α}-Boc-D-Pen Merrifield resin (substitution = 1.00 mmol/g). The first solid phase peptide synthesis (SPPS) coupling employed N^{α}-Boc-*p*-chloro-L-phenylalanine. A 1.3-g sample of the protected, resin-bound precursor peptide was treated with 12 mL of HF, 0.6 g of *p*-cresol, and 0.6 g of *p*-thiocresol. After extraction with 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptide was subjected to semipreparative HPLC, which yielded, after lyophilization, 135 mg of pure linear peptide. A sample of 40.0 mg of this peptide was subjected to oxidation as described above, yielding after RP-HPLC, 18.7 mg of disulfide-containing peptide.

Tyr-D-Cys-*p*-MePhe-D-PenOH (6). The linear compound was prepared using 1 g of *S*-*p*-MeBzl-N^{α}-Boc-D-Pen Merrifield resin (substitution = 1.20 mmol/g). The first coupling employed N^{α}-Boc-*p*-methyl-L-phenylalanine. A 1.3-g sample of the protected, resin-bound precursor peptide was treated with 11 mL of HF, 0.6 g of *p*-cresol, and 0.6 g of *p*-thiocresol. After extraction into 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptide was subjected to semipreparative HPLC, which yielded, after lyophilization, 94.6 mg of pure linear peptide. A sample of 44.5 mg of this peptide was subjected to oxidation as described above, yielding after RP-HPLC, 10.0 mg of cyclized peptide.

Tyr-D-Cys-*p*-*t*-BuPhe-D-PenOH (7a and 7b). The title peptides were prepared as was 2, by using N^{α}-Boc-4′-*tert*-butyl-D,L-phenylalanine in the first coupling. A 1.2-g sample of the protected, resin-bound precursor peptide was treated with 10 mL of HF, 0.6 g of *p*-cresol, and 0.6 g of *p*-thiocresol. After extraction from the resin with 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptides were subjected to semipreparative HPLC, which yielded after lyophilization, 2 pools of peptide, each enriched in one diastereomer, with respective masses of 52.0 and 63.0 mg. A 35.0-mg sample from the first pool and 33.0 mg from the second pool were subjected to oxidation as described above. The earlier-eluting disulfhydryl peptide yielded, after oxidation and RP-HPLC, 9.8 mg of cyclized peptide **7a**. After oxidation and RP-HPLC purification, the later-eluting linear peptide yielded 6.0 mg of disulfide-containing peptide **7b**.

Tyr-D-Cys-Tyr-D-PenOH (8). The title peptide was prepared as was 2, substituting N^{α}-Boc-L-tyrosine, without protection of the phenolic hydroxyl, in coupling 1. A 1.2-g sample of the protected, resin-bound precursor peptide was treated with 11 mL of HF, 0.6 g of *p*-cresol, and 0.6 g of *p*-thiocresol. After extraction with 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptide was subjected to semipreparative HPLC, which yielded, after lyophilization, 52.0 mg of pure linear peptide. A sample of 36.5 mg of this peptide was oxidized as described above, yielding after RP-HPLC, 8.9 mg of cyclized peptide.

Tyr-D-Cys-*m*-Tyr-D-PenOH (9a and 9b). The title peptides were prepared as was 2, by using N^{α}-Boc-D,L-*m*-tyrosine. A 1.3-g sample of the protected, resin-bound precursor peptide was treated

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with 11 mL of HF, 0.6 g of *p*-cresol, and 0.6 g of *p*-thiocresol. After extraction into 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptides were subjected to semipreparative HPLC, which yielded after lyophilization, 2 pools of diastereomeric peptide, each enriched in one component, with masses of 35.0 and 56.0 mg, respectively. In each case, the entire sample was subjected to oxidation as described above, and each product peptide required repeated RP-HPLC purification under isocratic conditions of 16% organic component on a (1.0 × 25)-cm column. The earlier-eluting disulfhydryl-containing, linear peptide yielded, after oxidation and extensive RP-HPLC, 4.9 mg of cyclized peptide **9a**. The later-eluting linear peptide yielded, after oxidation and extensive purification, 5.2 mg of disulfide-containing peptide **9b**.

Tyr-D-Cys-*p*-NO_2Phe-D-PenOH (10). The linear peptide was prepared as was **2**, by using N^{α}-Boc-*p*-nitro-L-phenylalanine. A 1.3-g sample of the protected, resin-bound precursor peptide was treated with 12 mL of HF, 0.6 g of *p*-cresol, and 0.6 g of *p*-thiocresol. After extraction into 9:1 DMF/80% HOAc and dilution with 0.1% TFA in H_2O, the disulfhydryl peptide was subjected to semipreparative HPLC, which yielded, after lyophilization, 189 mg of pure linear peptide. A sample of 45.0 mg of this peptide was subjected to oxidation as described above, yielding after RP-HPLC, 25.0 mg of disulfide-containing peptide.

General Methods for Peptide Analysis. Final product purity was determined by analytical RP-HPLC on a Vydac 218TP C-18 column (4.6 × 250 mm) by a gradient of 10–70% organic component over 30 min, with a flow rate of 1 mL/min. Peaks were monitored at 230 and 280 nm and analyzed with Waters Maxima 820 software. Peptide purity was assessed as >95% for each of the peptides reported here. All analytical RP-HPLC gradients were run by using the solvent system 0.1% TFA in water/0.1% TFA in acetonitrile. Peaks which also appeared in chromatograms in which no peptide was injected were considered to be artifacts and were ignored. Peptide purity was then evaluated by integration of peaks. In addition, all newly reported peptides were subjected to thin-layer chromatography (TLC) on precoated silica gel plates in three solvent systems: (solvent ratios are volume:volume) (A) 1-butanol/acetic acid/water (4:1:5, organic component only); (B) 1-butanol/water (containing 3.5% acetic acid and 1.5% pyridine) (1:1, organic component only); (C) *n*-amyl alcohol/pyridine/water (7:7:6). In all cases, a single spot was detected by using three methods of visualization (ninhydrin, UV absorption, iodine vapor) for each solvent system.

The absence of free sulfhydryl groups in final product peptides was confirmed by testing with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), which when combined with free sulfhydryl-containing species, forms an adduct which gives a characteristic yellow color and absorbance at 412 nm.[21] In all cases, absorbances of the final peptides at this wavelength were indistinguishable from sulfhydryl-free controls confirming the absence of free sulfhydryl groups.

^{1}H NMR spectra were registered on General Electric GN-500 and IBM WP 270 SY spectrometers, operating at 500 and 270 MHz, respectively. Samples contained 1–2 mg of the compound in D_2O, acidified D_2O, or DMSO, with 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionic acid sodium salt (TSP-d_4) added as the internal chemical shift standard. Diagnostic resonances originating from the methyl groups and the α proton of penicillamine and the aromatic resonances of tyrosine and phenylalanine confirmed the presence of these residues. All peptides also displayed NH-αCH-βCH_2 connectivities consistent with the presence of cysteine. No resonances were observed that could not be accounted for, and all resonances pertaining to specifically-modified residue 3 amino acids were present (peak patterns consistent with substituted aromatic rings). Some analogues were subjected to 2D COSY analysis to establish intraresidue connectivities and/or to 2D NOESY experiments to obtain primary sequences from interresidue NOE interactions. In each case, the anticipated sequence was confirmed.

Final product confirmation was obtained by fast atom bombardment mass spectrometry (FAB-MS). Since associated anions are not discerned by this method, the observed molecular weights, listed in Table II, correspond to the predicted values for the free peptides, rather than for the actual isolated trifluoroacetate salts.

Preparation of Guinea Pig Brain Homogenate. Ten frozen guinea pig brains (Pel-Freez Biologicals), weighing approximately 36 g, were thawed at 5 °C for 3 h. The brains were then suspended in cold 50 mM Tris buffer, pH 7.4, at a concentration of 0.1 g/mL (approximately 360 mL total) and homogenized for 20 s on a Brinkman Polytron homogenizer (Model PT 10-35, probe Model PT 20 ST) at one-half maximum speed. The homogenate was centrifuged at 14 000*g* for 15 min at 4 °C, and the supernatant was discarded. The pellets were resuspended in 360 mL of cold 50 mM Tris buffer by homogenization for 20 s at one-quarter maximum speed. Centrifugation at 4 °C was repeated at 14 000*g* for 15 min, and the supernatant was poured off. The pellets were again resuspended by homogenization (20 s at one-quarter speed) in 360 mL of 50 mM Tris buffer at 25 °C, pH 7.4. Following incubation at 37 °C for 30 min to release endogenous opioids, the homogenate was centrifuged at 14 000*g* for 15 min at 4 °C. Pellets of brain membrane finally were resuspended in 400 mL of 50 mM Tris buffer on ice, pH 7.4, by homogenization for 20 s at one-quarter speed. The membrane suspension was divided into aliquots of the appropriate size for a set of binding assays (approximately 10 mL) and stored in capped plastic test tubes at −80 °C. Before use in a binding assay, each aliquot was thawed, homogenized (eight strokes in a glass homogenizer with a Teflon pestle), and stored on ice.

Receptor Binding Assays. Receptor binding assays, done in triplicate, were performed as follows. In polypropylene tubes in an ice bath, assay components were carefully pipetted in the following order: 25 μL of water (total binding), displacing ligand in water (nonspecific binding), or test compound (varying concentrations prepared by serial dilutions) in water, and then 200 μL of membrane preparation (determined in control experiments to be in the linear range of the specific binding vs protein concentration dependence). These were gently vortexed and preincubated in a temperature-regulated water bath for 15 min at 25 °C, and then placed on ice for the addition of 25 μL of the radioligand solution in 50 mM Tris buffer (pH 7.4). The assay suspension in each tube was again gently vortexed and incubated in a water bath to reach equilibrium (75 min for assays using 0.6 nM [^{3}H]DAMGO and 1.8 nM [^{3}H]DPDPE, 90 min for assays using 0.9 nM [^{3}H]U69,593, each determined from time-dependence studies of specific binding) at 25 °C. Before harvesting the samples, the filter paper sheet was pretreated for 1 min with amyl alcohol-saturated water, followed by one Tris buffer wash. Subsequently, the samples were diluted, rapidly filtered, and washed twice with 4 mL of cold Tris buffer (pH 7.4), using a 24-tube Brandel cell harvester (Model M-24R). Filter discs were removed with forceps and placed in scintillation vials, to which 1 mL of ethanol and 10 mL of Scintiverse liquid scintillant were added. Capped vials were shaken by hand for 10 s and allowed to sit several hours. Radioactivity on the filters was then determined by liquid scintillation counting for 10 min per sample. Radioligand stock solution aliquots also were counted similarly after transfer into vials and addition of 10 mL of Scintiverse. Inhibition of radiolabeled ligand binding by the test compounds was computed from maximal specific binding, determined with an appropriate excess of unlabeled ligand (10 μM DPDPE or U69,593; 5 μM DAMGO). At the concentrations employed for the assays, total binding of each radioligand to the membrane preparation typically measured approximately 2000 dPM, while nonspecific binding was approximately 200 dPM for [^{3}H]DAMGO and [^{3}H]U69,593 and 400 dPM for [^{3}H]DPDPE. IC_{50} values were obtained by linear regression from plots relating inhibition of specific binding to the log of 12 different ligand concentrations, by using the computer program LIGAND[22] (Biosoft Software). K_i values were similarly calculated using values for K_D of each ligand, determined by analysis of saturation binding experiments. Values of K_D so determined were as follows: [^{3}H]DPDPE, K_D = 2.97 nM; [^{3}H]DAMGO, K_D = 0.85 nM. For binding to κ receptors, expected to be weak for all analogues, the protocol was altered to include

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only five ligand concentrations (in duplicate). Analysis by LIGAND, by using K_D = 0.9 nM for [^{3}H]U69,593, yielded estimates or lower limits for K_i.

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Analogues of the Muscarinic Agent 2′-Methylspiro[1-azabicyclo[2.2.2]octane-3,4′-[1,3]dioxolane]: Synthesis and Pharmacology

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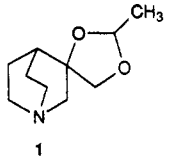
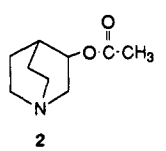
A number of tetrahydrofuran analogues of 2′-methylspiro[1-azabicyclo[2.2.2]octane-3,4′-[1,3]dioxolane] (1) have been prepared with the aim to obtain information about the relative importance of each of the oxygens in 1 for efficacy and for selectivity. In addition, the dimethyl and desmethyl analogues of 1 were prepared. The new compounds were compared to *cis*- and *trans*-1 with regard to their ability to displace (-)-[^{3}H]-3-quinuclidinyl benzilate ((-)-[^{3}H]QNB) from muscarinic receptors in cerebral cortex, heart, parotid gland, and urinary bladder from guinea pigs. Functioinal studies were made on isolated guinea pig bladder and ileum. The new compounds exhibited both lower affinity and efficacy than *cis*-1. A conformational study was performed, and the effects of steric and electronic factors on the biological activity of the compounds are discussed.

Introduction

The identification of a multitude of muscarinic receptor subtypes[1] has spurred the search for agonists and antagonists with selectivity for a given receptor subtype. Much interest has been focused on the development of selective muscarinic M1 agonists since such agents are of potential use in the therapy of Alzheimer type dementia and related disorders in which central cholinergic transmission is deficient.[2] However, little is known about the relationship between structure and subtype–receptor selectivity of agonists/antagonists. To further investigate the structural requirements for muscarinic agonist activity and subtype–receptor selectivity we have synthesized some analogues of 2′-methylspiro[1-azabicyclo[2.2.2]octane-3,4′-[1,3]dioxolane] (AF30; 1). Compound 1 was first synthesized in 1976 by Fisher et al.[3] as a rigid analogue of acetoxyquinuclidine (aceclidine; 2). The cis isomer of 1 was claimed to possess some selectivity toward M1 receptors as opposed to aceclidine. Fisher et al.[4] suggested that the selectivity was related to the low conformational flexibility of the compound.[5]



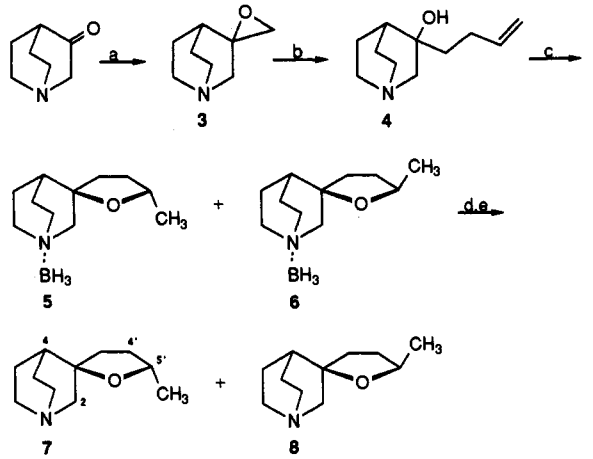
1



2

We have prepared a number of tetrahydrofuran analogues of 1 (7, 8, 14, and 15) with the aim to obtain information about the relative importance of each of the oxygens in 1 for efficacy and for selectivity. In addition,

Scheme I[a,b]



[a] Reagents: (a) trimethylsulfoxonium iodide, NaH, DMSO; (b) allylmagnesium bromide, CuI, ether; (c) (i) $Hg(OAc)_2$, THF/H_2O, (ii) $NaBH_4$, NaOH, benzyltriethylammonium chloride; (d) separation of diastereomers with flash chromatography; (e) HCl, MeOH. [b] Only relative stereochemistry is indicated.

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