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Synthesis and Biological Activities of YkFA Analogues: Effects of Position 4 Substitutions and Altered Ring Size on In Vitro Opioid Activity

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Abstract—Substitution in position 4 of the potent opioid peptide YkFA with aliphatic hydrophobic residues resulted in compounds that retained low nanomolar activities at both μ and δ opioid receptors, while ring contraction by incorporation of diaminobutyric acid in position 2 resulted in a more pronounced decrease in potency at both receptors for the ψ[CH_2NH] pseudopeptide as compared to the all amide parent.

There has been considerable interest in the development of peptide and peptidomimetic opioids since the discovery and pharmacological characterization of the first known endogenous opioid peptides, the enkephalins.[1] It is generally accepted that there are three opioid receptor subtypes (μ, δ, and κ)[2] and the endogenous ligands for each of these subtypes have been isolated and pharmacologically characterized.[3,4] While the physiological roles for each of the individual opioid receptors have not been clearly defined, recent evidence indicates that the μ opioid receptor mediates the analgesic properties of morphine as well as the development of dependence.[5,6] However, there is evidence that δ[7] and κ[8] receptor selective opioids may act as potent analgesics with a reduced propensity for eliciting some of the side effects associated with morphine use, including respiratory depression and the development of tolerance and dependence. Since the precise physiological roles of the opioid receptor subtypes are still unknown, there is a need for the development of more potent and receptor selective opioids for use in further pharmacological studies.

One approach to the development of potent and receptor specific peptides has been the use of conformational constraints.[9] This strategy has been applied extensively in the field of opioid peptides and has resulted in the development of highly selective compounds for both the μ and δ opioid receptors.[10] Cyclization has produced μ selective agonists such as Tyr-c[D-Dab-Gly-Phe-Leu][11] and Tyr-c[D-Orn-Phe-Asp]-NH_2[12] as well as selective μ opioid antagonists based upon a modified somatostatin analogue.[13] In addition, conformational constraint has been used in the development of the highly δ selective opioid receptor agonists DPDPE[14] and JOM-13,[15] both of which are cyclized via disulfide bonds. The conformationally constrained phenylalanine analogue Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) has been incorporated into a peptide first described by Schiller et al. known as TIPP.[16] The TIPP family of peptides includes examples of δ agonists, δ antagonists, and compounds with mixed μ agonist/δ antagonist activity that show promise as potential therapeutic agents.[17,18] It has also been shown by Salvadori et al. that the δ antagonism exhibited by TIPP and its analogues can be maintained in the Dmt-Tic dipeptide[19] and derivatives thereof.[20]

Darlak et al. first reported the synthesis and biological activities of the small ring cyclic peptide Tyr-c[D-Lys-Phe-Ala] (YkFA), which is cyclized through an amide bond between the side chain of D-Lys^2 and the carboxylate of Ala^4.[21] YkFA is a very potent opioid agonist at both μ and δ receptor subtypes as measured in the guinea pig ileum assay (IC_{50} = 0.19 nM) and the mouse vas

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deferens ($IC_{50} = 0.54$ nM), respectively. An extensive conformational analysis of YkFA has been performed, which incorporated dihedral angle and interproton distance constraints from NMR experiments with molecular modeling.[22] The resulting proposed bioactive conformation for μ receptor activity agreed very well with previously developed models for the μ receptor selective peptides Tyr-D-(NMe)Ala-Phe-D-Pro-NH_2 and Tyr-c[D-Orn-Phe-Asp]-NH_2. The NMR experiments indicated no clear preference between the *trans* and *gauche*$^-$ conformation in the Phe^3 side chain, resulting in two different models for the bioactive conformer at the δ receptor. The conformation in which the Phe^3 side chain is *trans* has an overall topographical arrangement of the pharmacophoric groups that is very similar to that proposed by Hruby et al. for the highly δ receptor selective peptide DPDPE.[23] The model of YkFA with the Phe^3 side chain in a *gauche*$^-$ conformation was found to be very similar to the δ active conformations proposed by Mosberg et al. for JOM-13.[24] These studies suggested specific topologies of YkFA responsible for its high affinity at both μ and δ opioid receptors. We have previously reported the synthesis and opioid activities of YkFA analogues that contained phenylalanine in position 1.[25] Two of these compounds retained moderate activity in the GPI but were more than twice as μ selective as the parent YkFA. These compounds are among a small group of opioid peptides that retain significant opioid activity even after the loss of the N-terminal phenolic hydroxyl group.[26–28] We report here further studies aimed at modulating the potency and receptor selectivity of YkFA through the substitution of Val, Leu, Ile, and Asp in position 4, and in the incorporation in two new analogues of α,γ-diaminobutyric acid (Dab) in position 2.

Peptide Synthesis and Purification

The compounds in this study were synthesized through a combination of solid-phase synthesis of a protected linear peptide and solution-phase cyclization in a manner similar to that previously described for YkFA.[25] Protected linear peptides were synthesized by stepwise elongation on Merrifield, hydroxymethyl, or Wang resin, using Boc or Fmoc strategies, with BOP/HOBt as the condensing agent. Protected linear peptides were cleaved using anhydrous HF (Merrifield and hydroxymethyl resin) or TFA (Wang resin). The reduced amide bond in **7** was introduced according to the method of Sasaki and Coy,[29] and *N*-α-Fmoc-*N*-γ-Boc-D-diaminobutyric acid (D-Dab in compounds **6** and **7**) was synthesized from Fmoc-D-Gln-OH via the 'acidic' Hoffman rearrangement according to a literature method.[30] The protected aspartic acid derivative Boc-Asp-OFm was anchored to hydroxymethyl resin through the side chain and cyclization was performed on the resin following a literature procedure to obtain **5**.[31]

Linear peptides were cyclized in solution under conditions of high dilution (10^{-3} M) and low temperature. The progress of the cyclization was monitored using analytical reverse-phase high performance liquid chromatography (RP-HPLC). After treatment with mixed-bed resin, the N-terminal Fmoc protecting group was cleaved with piperidine in DMF and the final compounds were purified using a combination of gel permeation chromatography (GPC) and RP-HPLC. Compound **5** was purified using GPC and cation exchange chromatography. The purity of the final compounds was assessed using analytical RP-HPLC with monitoring at both 220 and 254 nm and thin-layer chromatography (TLC) in three different solvent systems. The structural integrity was confirmed by amino acid analysis (D-Dab was not quantitated in **6** and **7**) and electrospray mass spectrometry (ES-MS) in the positive mode.

Results and Discussion

The opioid activities of the compounds in this study were determined using in vitro bioassays that rely upon the ability of opioids to inhibit the electrically induced contractions of the guinea pig ileum (GPI) and the mouse vas deferens (MVD).[32] Inhibition of muscle contraction is mediated primarily through the μ opioid receptor in the GPI and the δ opioid receptor in the MVD. The results of the in vitro bioassays are listed in Table 1. The data from Table 1 shows that the potency of compounds **2**–**4** is relatively insensitive to the increase in steric bulk that occurs upon replacement of Ala^4 with Val, Leu, and Ile. Compound **2** (with an isopropyl side chain) is essentially equipotent with YkFA, whereas the increased bulk and different branching patterns present in **3** and **4** result in an approximately 10-fold decrease in activity at both μ and δ opioid receptors relative to YkFA. While an order of magnitude less potent than YkFA, compounds **3** and **4** still retain low nanomolar potencies in both bioassays.

It has been suggested that a hydrophobic portion of molecules such as DPDPE and JOM-13 constitutes a fourth determinant for maintaining high levels of δ

Table 1. Bioassay results and μ selectivity of YkFA and analogues

	Peptide	$IC_{50} \pm SEM$ (nM)		Selectivity
		GPI (μ)	MVD (δ)	IC_{50} (δ)/IC_{50} (μ)
1	Tyr-c[D-Lys-Phe-Ala]	0.11±0.013	0.54±0.031	4.9
2	Tyr-c[D-Lys-Phe-Val]	0.20±0.02	0.44±0.01	2.2
3	Tyr-c[D-Lys-Phe-Leu]	1.4±0.4	6.2±2.4	4.5
4	Tyr-c[D-Lys-Phe-Ile]	1.1±0.3	6.3±2.2	6.0
5	Tyr-c[D-Lys-Phe-Asp]	78.8±15.6	73.8±30.0	0.94
6	Tyr-c[D-Dab-Phe-Ala]	3.5±1.3	40.0±13.9	12
7	Tyr-c[D-Dab-Pheψ[CH_2NH]Ala]	21.4±6.6	252±47	12

opioid potency and selectivity.[33] Our initial assumption that the side chain of the position 4 amino acid in YkFA may correspond to this hydrophobic region seems unlikely in view of the potent, but nonselective analogues that incorporate valine, leucine, and isoleucine. A direct comparison of the previously developed models for the δ opioid receptor-active conformations of YkFA,[22] DPDPE,[23] and JOM-13[24] was then performed using Insight II, version 97.0. These comparisons indicated that excellent overlap can be otained between the tyramine and phenyl pharmacophoric groups of YkFA and the corresponding moities of DPDPE and JOM-13, resulting in overlap of the hydrophobic disulfide bridge in DPDPE and JOM-13 with the side-chain methylenes of D-Lys^2 in YkFA. Thus, it seems likely that the side chain of D-Lys^2 in YkFA corresponds to this hydrophobic determinant, and not the side chain of the position 4 amino acid.

The substitution of aspartic acid (**5**) for alanine in position 4 of YkFA resulted in a much more dramatic decrease in opioid activities than the conservative Val, Leu, and Ile substitutions. Compound **5** most closely resembles a truncated analogue of the δ selective peptide deltorphin I (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2), however **5** is equipotent in the MVD and GPI assays. The loss in activity at the μ receptor is not surprising in light of structure–activity studies that suggest the carboxylate side chain of aspartic acid in deltorphin I is involved in a direct repulsion with the binding site on this receptor.[34] Apparently, the negative charge on this residue is incompatible with the topography of the μ receptor, while the δ receptor can accommodate not only the anionic carboxylate of Asp in deltorphin I, but the cationic His^4 present in the δ-selective opioid deltorphin (Tyr-D-Ala-Phe-His-Leu-Met-Asp-NH_2).

Molecular modeling provides further insights into the putative δ receptor active conformation of analogue **5**. Starting with the previously developed models for the δ receptor active conformation of YkFA, the side chain of Ala^4 was replaced with the carboxylate of aspartic acid and the resulting structure was minimized. While similar modeling of compounds **2**–**4** indicated that the Val, Leu, and Ile substitutions at position 4 were compatible with the previously proposed δ conformations, the aspartic acid substitution results in a disruption of the bioactive conformation.

The original work noted that the δ active conformations were stabilized by hydrophobic interactions between the aromatic rings of Tyr^1 and Phe^3 and the side chain of Ala^4, which form a hydrophobic face.[14] Thus, it is not surprising that our modeling indicates that the Val, Leu, and Ile substitutions are compatible with conformers of position 1, 3, and 4 side chains as is seen in YkFA.

Further, the introduction of a negatively charged carboxylate at position 4 might be expected to disrupt these hydrophobic interactions, and significantly disturb the conformation of that face of the molecule. More specifically, modeling indicates that the cyclic backbone is not disturbed, but the aromatic ring of Tyr^1 is clearly shifted (relative to YkFA) closer to the side chain of Asp^4. This brings the hydroxyl and carboxylate groups into proximity for a possible hydrogen bonding interaction which lowers the energy of this conformer relative to YkFA. Another consequence is the rotation of the aromatic ring of Phe^3 (relative to YkFA) so as to increase the distance between the polar carboxylate of Asp^4 and the hydrophobic benzene ring. Because the benzene ring of Phe^3 is thought to fit into a site on the δ receptor that is flat, any rotation of the plane of the aromatic ring could result in a less than optimal fit, lowering the activity for the Asp^4 analogue at this receptor. However, it seems likely that free rotation about the χ^2 angle could result in the aromatic ring assuming the proper orientation for a favorable interaction with a minimal energy expenditure.

Although YkFA is constrained by cyclization between the side chain of D-Lys^2 and the carboxylate of Ala^4 it is still capable of facile interconversion between the two different conformations required for binding and signal transduction at the μ and δ opioid receptors. In an effort to increase the conformational constraint within this small cyclic peptide we have synthesized two analogues (**6** and **7**) in which D-Lys^2 has been replaced with α,γ-diaminobutyric acid (Dab), a lysine analogue that contains two methyene groups in its side chain. We reasoned that the 4 methylene groups in the side chain of D-Lys^2 were a source of residual flexibility within YkFA and that by removing two of these groups and decreasing the flexibility of the cyclic portion of the molecule we may decrease the interconversion between the μ and δ active conformations, thereby possibly increasing selectivity.

As the data in Table 1 shows, the contraction of the ring from 13 to 11 atoms in **6** results in a compound that has decreased potency in the GPI ($IC_{50} = 3.5$ nM) relative to YkFA. A larger decrease in activity at the δ receptor however makes compound **6** more than twice as selective as YkFA. The introduction of a reduced amide bond between Phe^3 and Ala^4 in the smaller ring system (**7**) causes a similar decrease in potency at both receptor types (approximately 6-fold). Thus, compound **7** is less potent than **6** in both assays, but has a similar selectivity ratio. It is possible that the Phe^3 carbonyl group, which is capable of acting as a hydrogen bond acceptor, is involved in an interaction with the opioid receptors that is important in maintaining potency. Alternatively, the increased flexibility of the reduced amide bond in **7** may be responsible for the drop in potency by allowing the molecule to assume conformations that do not complement the receptor binding sites. Together, these results indicate that the larger ring system present in YkFA may allow the molecule to adopt conformations that more closely complement the topography of the receptor binding sites. Thus, the 13-membered ring system of YkFA may represent a better template for the design of more potent and selective analogues.

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References and Notes

1. Hughes, J.; Smith, T. W.; Kosterlitz, H. W.; Fothergill, L. A.; Morgan, B. A.; Moriss, H. R. *Nature* **1975**, *258*, 577.
2. Lord, J. A. H.; Waterfield, A. A.; Hughes, J.; Kosterlitz, H. W. *Nature* **1977**, *267*, 495.
3. Höllt, V. *Annu. Rev. Pharmacol. Tox.* **1986**, *26*, 59.
4. Zadina, J. E.; Hackler, L.; Ge, L.-J.; Kastin, A. J. *Nature* **1997**, *386*, 499.
5. Matthes, H. W. D.; Maldonado, F. S.; Valverde, O.; Slowe, S.; Kitchen, I.; Befort, K.; Dierich, A.; Le Meur, M.; Dolle, P.; Tzavara, E.; Hanoune, J.; Roques, B. P.; Kieffer, B. *Nature* **1996**, *383*, 819.
6. Sora, I.; Takahashi, N.; Funada, M.; Ujike, H.; Revay, R. S.; Donovan, D. M.; Miner, L. L.; Uhl, G. R. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 1544.
7. Freye, E.; Schnitzler, M.; Schenk, G. *Pharm. Res.* **1991**, *8*, 196.
8. Narita, M.; Funada, M.; Suzuki, T. *Pharmacol. Ther.* **2001**, *89*, 1.
9. Hruby, V. J.; Al-Obeidi, F.; Kazmierski, W. *Biochem. J.* **1990**, *268*, 249.
10. Hruby, V. J.; Gehrig, C. A. *Med. Res. Rev.* **1989**, *9*, 343.
11. DiMaio, J.; Schiller, P. W. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 7162.
12. Schiller, P. W.; Nguyen, T. M.-D.; Lemieux, C.; Maziak, L. A. *J. Med. Chem.* **1985**, *28*, 1766.
13. Pelton, J. T.; Gulya, K.; Hruby, V. J.; Duckles, S. P.; Yamamura, H. I. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 236.
14. 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.
15. Mosberg, H. I.; Omnaas, J. R.; Medzihradsky, F.; Smith, C. B. *Life Sci.* **1988**, *43*, 1013.
16. Schiller, P. W.; Nguyen, T. M.-D.; Weltrowska, G.; Wilkes, B. C.; Marsden, J.; Lemieux, C.; Chung, N. N. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 11871.
17. Schiller, P. W.; Fundytus, M. E.; Merovitz, L.; Weltrowska, G.; Nguyen, T. M.-D.; Lemieux, C.; Chung, N. N.; Coderre, T. J. *J. Med. Chem.* **1999**, *42*, 3520.
18. Schiller, P. W.; Weltrowska, G.; Berezowska, I.; Nguyen, T. M.-D.; Wilkes, B. C.; Lemieux, C.; Chung, N. N. *Biopolymers (Peptide Sci.)* **1999**, *51*, 411.
19. Salvadori, S.; Attila, M.; Balboni, G.; Bianchi, C.; Bryant, S. D.; Crescenzi, O.; Guerrini, R.; Picone, D.; Tancredi, T.; Temussi, P. A.; Lazarus, L. H. *Mol. Med.* **1995**, *1*, 678.
20. Santagada, V.; Balboni, G.; Caliendo, G.; Guerrini, R.; Salvadori, S.; Bianchi, C.; Bryant, S. D.; Lazarus, L. H. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2745.
21. Darlak, K.; Burks, T. F.; Wire, W. S.; Spatola, A. F. In *Peptides 1990: Proceedings of the 21st European Peptide Symposium*; Giralt, E., Andreau, D., Eds.; ESCOM: Leiden, 1991; p 401.
22. Ro, S.; Zhu, Q.; Lee, C.-W.; Goodman, M.; Darlak, K.; Spatola, A. F.; Chung, N. N.; Schiller, P. W.; Malmberg, A. B.; Yaksh, T. L.; Burks, T. F. *J. Peptide Sci.* **1995**, *1*, 157.
23. Hruby, V. J.; Kao, L.-F.; Pettitt, B. M.; Karplus, M. *J. Am. Chem. Soc.* **1988**, *110*, 3351.
24. Mosberg, H. I.; Lomize, A. L.; Wang, C.; Kroona, H.; Heyl, D. L.; Sobczyk-Kojiro, K.; Ma, W.; Mousigian, C.; Porreca, F. *J. Med. Chem.* **1994**, *37*, 4371.
25. Burden, J. E.; Davis, P.; Porreca, F.; Spatola, A. F. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3441.
26. Mosberg, H. I.; Ho, J. C.; Sobczyk-Kojiro, K. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2681.
27. McFadyen, I. J.; Sobczyk-Kojiro, K.; Schaefer, M. J.; Ho, J. C.; Omnaas, J. R.; Mosberg, H. I.; Traynor, J. R. *J. Pharmacol. Exp. Ther.* **2000**, *295*, 960.
28. Schiller, P. W.; Berezowska, I.; Nguyen, T. M.-D.; Schmidt, R.; Lemieux, C.; Chung, N. N.; Falcone-Hindley, M. L.; Yao, W.; Liu, J.; Iwama, S.; Smith, A. B.; Hirschmann, R. *J. Med. Chem.* **2000**, *43*, 551.
29. Sasaki, Y.; Coy, D. H. *Peptides* **1987**, *8*, 119.
30. Loudon, G. M.; Radhakrishna, A. S.; Alsmond, M. R.; Blodgett, J. K.; Boutin, R. H. *J. Org. Chem.* **1984**, *49*, 4272.
31. Romanovskis, P.; Spatola, A. F. *J. Peptide Res.* **1998**, *52*, 356.
32. Kosterlitz, H. W.; Lord, F. A. H.; Paterson, S. J.; Waterfield, A. A. *Br. J. Pharmacol.* **1980**, *68*, 333.
33. Nikiforovich, G. V. *Lett. Pep. Sci.* **1995**, *2*, 172.
34. Melchiorri, P.; Negri, L.; Falconieri-Erspamer, G.; Severini, C.; Corsi, R.; Soaje, M.; Erspamer, V.; Barra, D. *Eur. J. Pharmacol.* **1991**, *195*, 201.