AN ABSTRACT OF THE THESIS OF

Sandhya Nitin Kulkarni for the degree of Master of Science in Pharmacy (Medicinal Chemistry) presented on June 5, 1995.

Title: The Synthesis and Opioid Activity of Dynorphin Analogues with Modifications in the Message Sequence.

Abstract approved:

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Jane V. Aldrich

This research was directed towards investigating the structure-activity relationships of dynorphin A (Dyn A) with the modifications in the N-terminal tetrapeptide “message” sequence. We synthesized a series of analogues of Dyn A to study the effect of modifications of this message sequence on opioid activity and receptor selectivity.

A tetrapeptide TIPP (Tyr-Tic-Phe-Phe-OH) and tripeptide TIP (Tyr-Tic-Phe) (where Tic = 1,2,3,4-tetrahydroisoquinoline-carboxylic acid) show selective δ-receptor antagonist properties. Therefore we combined the TIPP “message” sequence with the remaining “address” sequence of Dyn A and synthesized a series of analogues by solid phase peptide synthesis using the Fmoc synthetic strategy. We further modified the TIPP sequence by substituting Tic² by its structural analogues Pip and N-MePhe and also by substituting Phe³ by D-Phe, Gly and des-Gly (deletion of residue 3) to determine the effect on opioid activity. Opioid receptor affinities were evaluated in radioligand binding assays using cloned opioid receptors and opioid activity determined in the guinea pig il-
The modifications increased κ-receptor affinity compared to the control Phe² compounds in each series and preliminary data suggest that selected compounds may be lead compounds for the future development of kappa antagonists (Chapter 3). Further testing to confirm these results is in progress.

Since coupling of hindered amino acid residues using standard reagents (DIC [diisopropylcarbodiimide]/HOBt [1-hydroxybenzotriazole]) is often inefficient and can result in several side products, the usual synthetic strategy was modified for the last two couplings and PyBOP (benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate) was used to couple these residues. Still the crude peptides showed considerable amounts of side products due to incomplete couplings which were characterized by liquid chromatography in conjunction with electrospray mass spectral analysis. In order to minimize these side products and to improve the yields of the desired peptides, we selected a model peptide ([Tic²,Phe³,D-Pro¹⁰]Dyn A-(1-11)OH) and conducted a comparison study of different activating agents (PyBOP, PyBroP [bromotripyrolidinophosphonium hexafluoride] and HATU [O-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate]), efficient in coupling of hindered amino acids, and also compared the effects of coupling additives (HOBt vs. HOAt [1-hydroxy-7-azabenzotriazole]). Our study showed that HOAt was a better additive than HOBt since it reduced coupling times and also reduced undesirable side products. HATU and HOAt together gave the highest purity of the desired peptide and the coupling times were shortest. A comparison of syntheses was performed on a PAC® polystyrene (PS) resin and PEG (polyethylene glycol)-polystyrene (PEG-PS) resin. Our resin study with PS and PEG-PS showed almost no difference in the products produced and their proportions, but the latter resin gave slightly
shorter coupling times. The results of comparison of resins and coupling agents are dis­
cussed (Chapter 4).
Synthesis and Opioid Activity of Dynorphin Analogues
with the Modifications in the Message Sequence

by

Sandhya N. Kulkarni

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Sandhya Nitin Kulkarni, Author
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It was my greatest desire to complete my research projects successfully and also to obtain a degree at graduate level. Sometimes all of this almost looked like a dream! But it was possible because of few people and institutions, who deserve my greatest recognition and I don’t think I would have achieved my goal without them.

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NON-STANDARD ABBREVIATIONS

BOP  (benzotriazolyloxy)tris (dimethylamino) phosphonium hexafluorophosphate
DCM  dichloromethane
DIC  diisopropylcarbodiimide
DIPEA diisopropyl ethyl amine
DMA  N,N-dimethylacetamide
EDT  ethane dithiol
EKC  ethylketocyclozocine
FAB-MS  fast atom bombardment mass spectrometry
Fmoc  9-fluorenyl methoxy carbonyl
HOBt  1-hydroxybenzotriazole
HPLC  high performance liquid chromatography
HOAT  1-hydroxy-7-azabenzotriazole
HATU  O-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate;
MBHA  4-methylbenzahydrylamine
MVD  mouse vas deferens
Pmc  2,2,5,7,8-pentamethylchroman-6-sulfonyl
PyBOP benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate
<table>
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<th>Abbreviation</th>
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<tr>
<td>PyBroP</td>
<td>bromotripyridinophosphonium hexafluoride</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>Tic</td>
<td>1, 2, 3, 4 Tetrahydro-iso-quinoline-3-carboxylic acid</td>
</tr>
<tr>
<td>Pip</td>
<td>pipecolic acid</td>
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CHAPTER 1

INTRODUCTION

The analgesic activity of opium and its clinical use as a pain killer has been known for thousands of years (Evans et al., 1988). This oldest painkiller in the world, extracted from the juice of *Papaver somniferum*, had an important place in Greek, Roman and Arabic medicine. However, opium’s main ingredient morphine is associated with the problems of addiction, abuse potential and also other undesirable side effects (respiratory and pulmonary depression, constipation, etc.). There are limitations on the medicinal use of morphine and its derivatives since they produce physical and psychological dependence and tolerance. This initiated the search for potent non-addictive analgesics without these undesirable side effects.

Initially many attempts were made to develop drugs with similarity to the basic structural skeleton of morphine. It was observed that slight changes in the side chain of the morphine molecule considerably affected the potency of analgesia. However, the central problem of opiate pharmacology remains to be solved, namely the development of a pain killing drug with morphine's analgesic potency but without its negative side effects.

A notable discovery in the field of opioids came in 1975, when endogenous opiate receptor ligands, the enkephalins (meaning “in the head”), were found in brain extracts
Enkephalins are peptides and not alkaloids like morphine. Also three major types of opioid receptors ($\mu$, $\delta$, $\kappa$) have been identified which were characterized by their prototype agonists morphine, enkephalins and ketocyclazocine (Martin et al., 1976, Lord et al., 1977).

Dynorphin A (Dyn A), a potent endogenous opioid peptide, was first isolated and identified from porcine pituitary (Cox et al., 1975, Goldstein et al., 1979). Dyn A interacts preferentially with $\kappa$ receptors and so has been postulated to be an endogenous $\kappa$ receptor ligand (Chavkin et al., 1981). It has also been proposed that $\kappa$ receptor selective ligands have lower potential for abuse, addiction, tolerance and respiratory depressant effects compared to morphine and its structural analogues (Jaffe et al., 1990, Wolleman et al., 1993). Therefore it is important to develop more potent and $\kappa$ receptor selective ligands to study the physiological roles of dynorphin A.

Our research group is mainly involved in investigating the structure-activity relationships of Dyn A and their effects on opioid activity as well as receptor selectivity. Dyn A, a heptadecapeptide (17-amino acid residues), can be divided into two distinct regions, the N-terminal tetrapeptide "message" sequence and the remaining C-terminal "address" sequence (Chavkin et al., 1982). The "message" sequence is important for the opioid activity of Dyn A (Chavkin and Goldstein, 1981). In order to explore this concept further, we decided to modify the tetrapeptide "message" sequence.

The first project of this thesis involves the synthesis of a series of [D-Pro$^{10}$]Dyn A (1-11)OH analogues with modifications in the "message" sequence by solid phase peptide synthesis. The peptides were assembled on a hydroxymethylphenoxyacetic acid
(PAC®) support using the Fmoc synthetic strategy. The tetrapeptides TIPP (Tyr-Tic-Phe-Phe) and tripeptide TIP (Tyr-Tic-Phe) (Fig. 4.1) synthesized by Schiller and coworkers show promising δ-antagonist properties (Schiller et al., 1993). Therefore we selected the TIPP sequence as a model for the "message" sequence and combined it with the "address" portion of Dyn A, in an attempt to develop κ-receptor antagonists. Since both Tyr¹ and Phe⁴ are believed to be essential for opioid activity (Chavkin and Goldstein, 1981), our further modifications were restricted to positions 2 and 3. The substitution of Tic² by Pip (pipelicolic acid) and NMePhe (N-methylphenylalanine) (Fig. 3.1) was the next step taken in modification of the tetrapeptide "message" fragment. Further substitution of Phe³ by D-Phe, Gly, or des-Gly (deletion of residue 3) led to the synthesis of different series of analogues. We also synthesized control compounds for this series with Phe² in place of Tic/Pip/N-MePhe and the same substitutions in position 3. Opioid receptor affinity was examined in radioligand binding assays and opioid activity determined in the electrically stimulated guinea pig ileum (GPI) (Chapter 3).

During the solid phase synthesis of these peptides, we modified our regular coupling strategy [DIC (diisopropylcarbodiimide) /HOBt (1-hydroxybenzotriazole)], anticipating difficulty in coupling the hindered amino acid residues. A recently introduced and promising phosphonium coupling reagent (Coste et al., 1990), PyBOP (Fig. 2.15), was used for the last two couplings. But still the crude peptides showed considerable amounts of several impurities due to incomplete couplings.

This encouraged us in the second project to conduct two comparison studies. For these studies we selected one of our peptides as a model peptide, {[Tic²,Phe³,D-Pro¹⁰]-
Dyn A (1-11)OH}. In the first part we conducted a comparison study of resins by assembling the model peptide on polystyrene (PS) and PEG (polyethylene glycol)-polystyrene resin (PEG-PS) respectively by solid phase synthesis using the Fmoc protocol (Chapter 4). PEG-PS was chosen for comparison to PS since it gives a higher level of solvation for the peptide chain which can increase the coupling efficiency. In the second part we performed a comparative study with the same model peptide, using different activating agents (PyBOP, PyBroP, HATU) (Fig. 2.15a) and coupling additives (HOBr vs HOAt) (Fig. 2.15b). All these reagents have been reported to increase the coupling efficiency, minimize the formation of side products and improve the yield of the desired peptides containing hindered amino acids (Chapter 4).

The results of all these studies are presented below.
2.1. The Opiate Receptors

The first model of opiate receptors was proposed by Beckett and Casy based on structure-activity relationships of narcotics (Beckett and Casy, 1954). Multiplicity of opioid receptors was first proposed by Portoghese (Portoghese, 1965). Martin, after studies with several opioid agonists, proposed the heterogeneity of opioid receptors (Martin et. al., 1976). He suggested the presence of three different types of receptors, mu (μ), kappa (κ) and sigma (σ) receptors, with the prototype agonists for each receptor type as morphine (μ), ketocyclazocine (κ) and SKF 10047 (N-allylnormetazocine) (σ), respectively. The σ receptor is not considered to be an opioid receptor, since the pharmacological effects are not reversed by the opioid antagonist naloxone (Vaupel, 1983). An additional opioid receptor, the delta (δ) receptor, was discovered by Lord (Lord et al., 1977). Therefore μ, κ, and δ are the three main receptors accepted as opioid receptors.

The affinities of the endogenous opioid peptides for the different opioid receptors have been examined. Endogenous opioid peptides enkephalins were found to preferentially interact with δ receptors. Dynorphin A (Dyn A) shows some selectivity for κ-receptors and therefore it has been postulated to be an endogenous ligand for the κ receptors (Fig. 2.1). There is still uncertainty about the endogenous ligand for μ receptors.
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<tr>
<td><strong>Delta (δ)</strong></td>
<td>Enkephalins</td>
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<tr>
<td><strong>Kappa (κ)</strong></td>
<td>Dynorphin</td>
<td>Ketocyclazocine</td>
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Morphine:

![Morphine structure](image)

DADLE: [D-Ala²,D-Leu³]enkephalin

Ketocyclazocine:

![Ketocyclazocine structure](image)

Fig. 2.1. Various Prototypical Ligands for Opioid Receptors  
2.1.1. Cloned Opioid Receptors

Recently the three major types of opioid receptors (μ, κ, δ) have been cloned and functionally characterized. The first successful attempts were made by Evans (Evans et al., 1992) and Kieffer (Kieffer et al., 1992) when they cloned a mouse δ receptor. The search continued in this field and subsequently Yasuda and his group (Yasuda et al., 1993) successfully cloned δ and κ receptors. They found that the sequence of the cloned receptors had seven hydrophobic putative transmembrane domains and belong to the superfamily of G-protein coupled receptors (Yasuda et al., 1993). These findings were supported by a study by Kong (Kong et al., 1994). Kong and coworkers also found that there is about 60% amino acid identity among the sequences of the putative membrane-spanning segments of these receptors. Specifically sequence conservation is observed in the second and third transmembrane domains (Chen et al., 1993). Also it is proposed that there are different domains for binding of κ opioid agonists and antagonists (Kong et al., 1994).

2.1.2. Kappa Opioid Receptors

The distribution of κ-receptors show large differences between species. Kappa opioid receptors, proposed by Martin (Martin et al., 1976), were shown to be abundant in guinea pig brain (Magnan et al., 1979). The relative proportions of the binding sites in guinea pig brain are 44% κ, 24% μ and 32% δ (Gillan and Kosterlitz, 1982). In humans the proportion of κ-receptors is highest in the hypothalamus (Itzhak et al., 1982). The
proportion of κ-binding sites in rat brain, however, is only 12% (Gillan and Kosterlitz, 1982). The rabbit vas deferens seems to contain very high levels of κ-receptors (Oka et al., 1981). The guinea pig ileum also has a significant population of κ-receptors (Goldstein, 1983).

Further experimental evidence for the existence of κ-receptors was obtained by using a non-selective irreversible opioid antagonist, β-chlornaltrexamine (β-CNA) (James et al., 1982), and the μ-selective alkylating agent, naltrexone fumarate methyl ester (β-FNA) (Huidobro-Toro et al., 1982) when the GPI was treated with β-CNA with a small amount of Dyn A. Dyn A and ethylketocyloczocine (EKC) still showed high potency following the treatment with β-CNA, but the μ and δ selective ligands had no effect on potency (Chavkin et al., 1982). This suggested that dynorphin could selectively protect the alkyl- ation of kappa receptors by β-CNA, while μ and δ receptor sites were almost completely destroyed. Similarly β-FNA (a selective μ-receptor alkylating agent) decreased the opioid activity of only μ-agonists, while little change was observed in the potency of Dyn A or EKC.

2.1.3. Kappa Receptor Ligands

2.1.3.1. Kappa Receptor Agonists

The extensive search for κ-receptor selective ligands has resulted in the development of many κ-receptor agonists. Ethylketocyloczocine, bremozocine, tifluadom and MR
2034 have high affinity for κ-receptors, but low κ-receptor selectivity. U-50,488 was the first κ-selective agonist developed (Lathi et al., 1985). Subsequently its analogues, U-69,593, U-62,066, PD-117302 and ICI-199441, were prepared which are more potent and possess very high selectivity (Fig. 2.2)

κ-Receptor Agonists

![Chemical structures of kappa receptor agonists](image)

**Fig. 2.2. Kappa Receptor Agonists**
2.1.3.2. Kappa Receptor Antagonists

Naloxone was the first pure opioid antagonist which binds to all three major types of opioid receptors, with maximum affinity for \( \mu \) receptors (Lord et al., 1977). It is widely used to determine whether agonist activity is mediated through opioid receptors (Leslie, 1987). The antagonists MR-2266 and quadazocine (WIN-44441) have very weak \( \kappa \)-receptor selectivity, but antagonists developed by Portoghese and his group, namely TENA and nor-BNI, are highly \( \kappa \)-receptor selective (Fig. 2.3).
Fig. 2.3. Kappa-Receptor Antagonists
2.2. Opioid Peptides

2.2.1. The Endogenous Opioid Peptides

The discovery of the enkephalins leucine enkephalin (Leu-enkephalin) and methionine enkephalin (Met-enkephalin) by Kosterlitz and colleagues (Hughes et al., 1975) was followed rapidly by the isolation, characterization and identification of other endogenous opioid peptides, specifically the endorphins and dynorphins in mammals (Holt, 1986).

2.2.2. Precursor Proteins

The three precursor proteins for mammalian opioid peptides are proenkephalin, proopiomelanocortin (POMC), and prodynorphin which yield endogenous opioid peptides following proteolytic cleavage (Fig.2.5). Proenkephalin is the source for Met-enkephalin, two extended Met-enkephalins and Leu-enkephalin (Simon, 1991). POMC yields mainly β-endorphin. It is also the source for adrenocorticotropic hormone (ACTH) and γ-melanocyte stimulating hormone (γ-MSH) (Evans et al., 1988). Prodynorphin or (proenkephalin B) is the source of dynorphin A and dynorphin B and as well as α- and β-neoendorphin.
<table>
<thead>
<tr>
<th>Endogenous Mammalian Peptide</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Dynorphin A-(1-9)</td>
<td>H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-OH</td>
</tr>
<tr>
<td>• Dynorphin B (Rimorphin)</td>
<td>H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr-OH</td>
</tr>
<tr>
<td>• βν-Endorphin</td>
<td>H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH</td>
</tr>
<tr>
<td>• [Leu]Enkephalin</td>
<td>H-Tyr-Gly-Gly-Phe-Leu-OH</td>
</tr>
<tr>
<td>• [Met]Enkephalin</td>
<td>H-Tyr-Gly-Gly-Phe-Met-OH</td>
</tr>
<tr>
<td>• α-Neoendorphin</td>
<td>H-Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys-OH</td>
</tr>
<tr>
<td>• β-Neoendorphin</td>
<td>H-Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-OH</td>
</tr>
</tbody>
</table>

Fig. 2.4. Structure of Endogenous Opioid Peptides

Abbreviations used in the fig.: DYN = dynorphin, ENK = enkephalin, END = endorphin.

Fig. 2.5. Precursors of Opioid Peptides: A Schematic Presentation

2.2.3. Dynorphin A

Dynorphin A (Dyn A) was first isolated and identified from porcine pituitary (Cox et al., 1975). This potent opioid peptide interacts preferentially with κ receptors and so has been postulated to be an endogenous κ ligand (Chavkin et al., 1981). Dyn A is a heptadecapeptide (17-amino acid residues) which can be divided into two regions (Fig.2.6), the N-terminal tetrapeptide “message” sequence and the remaining C-terminal address sequence (Chavkin et al., 1982).

2.2.3.1. Structural Analysis of Dynorphin A

Structural analysis of biomolecules was mainly done by x-ray crystallography during the 1960’s and 1970’s. Recently other techniques, such as high resolution nuclear magnetic resonance (NMR) spectroscopy, Fourier transform infrared spectroscopy (FT-IR), Raman spectroscopy and circular dichroism (CD), have resulted in significant advancements in understanding the conformations of many peptides. However, there is contradictory evidence for the conformation of Dyn A using these techniques. A circular dichroism study in aqueous solution indicated that Dyn A assumes a random coil or β-sheet structure (Vaughn and Taylor, 1989). In the presence of trifluoroethanol (TFE) Dyn A shows negligible α-helical structure, whereas it assumes a significant α-helical structure in the presence of sodium dodecyl sulfate (SDS) (Maroun et al., 1985). Raman and FT-IR studies in aqueous or methanolic solution predicted that Dyn A is a mixture of extended and random structures (Rapaka, et al., 1987, Renugopalkrishnan, et al., 1988). Schiller
used fluorescent spectroscopy with a Trp-containing Dyn A analogue and predicted an extended structure in aqueous solution (Schiller, 1983). Schiller also demonstrated that the distance between Tyr\(^1\) and Trp\(^{14}\) in Dyn A is at least 20 °A in aqueous solution.

Fig. 2. Schematic Representation of the Dynorphin A Receptor

2.2.3.2. Model of Lipid Membrane Effects on Peptide Conformation

Lipids are believed to play an important role in the interaction of peptides with receptors. Several groups have studied the role of lipids in ligand-receptor interaction and Schwyzer’s group proposed an α-helical structure from Tyr\textsuperscript{1} to Pro\textsuperscript{10} of Dyn A (1-13) (Erne, et al., 1985). He proposed that Tyr\textsuperscript{1} to Leu\textsuperscript{5} are in contact with the hydrophobic phase, whereas Arg\textsuperscript{6} through Lys\textsuperscript{13} are in contact with the aqueous phase (Fig. 2.7). It was proposed that the segment Pro\textsuperscript{10}-Lys\textsuperscript{11}-Leu\textsuperscript{12}-Lys\textsuperscript{13} remains in a random conformation (Schwyzer, 1986, Schwyzer, 1992).

Fig. 2.7. Model of Dynorphin A-(1-13)
Schwyzer also proposed that the receptor selectivity of opioid peptides could be due to peptide-membrane interactions (Schwyzer, 1986). He proposed that the three major opioid receptors, μ, δ and κ receptors, must be located in different areas of the lipid bilayer membrane, as shown in Fig. 2.8.

Fig. 2.8. A Schematic Model of the “Three Compartment Model”
2.2.4. Metabolism of Dynorphin A

Dyn A can be metabolized to shorter fragments by cleavage at several sites by the action of various peptidases. The resulting fragments may or may not be active. This metabolism can complicate experiments for determining opioid activity and receptor binding affinity. However, the degradation of Dyn A can be controlled by lowering the incubation temperature or the use of peptidase inhibitors like bestatin (Leslie, 1982, Marks et al., 1986). It has been shown that Dyn A-(1-17) and Dyn A-(1-13) are less prone to degradation than Dyn A-(1-8) (Corbett, et al., 1982). There are certain positions in Dyn A at which the enzymes can attack and cleave the peptide (Fig.2.9).

N-Terminal degradation occurs mainly due to the action of aminopeptidase, diaminopeptidase and "enkephalinase" and yields inactive fragments. Tyr\(^1\) can be released by the action of membrane-bound aminopeptidase (Guyon et al., 1979), whereas the fragment Tyr-Gly can be cleaved by the enzyme diaminopeptidase (Gorenstein and Snyder, 1979). "Enkephalinase" (metalloendopeptidase EC 3.4.24.11) yields the Tyr-Gly-Gly fragment (Malfroy et al., 1978).

C-Terminal degradation occurs due to metabolism by carboxypeptidase and enkephalinase and yields inactive fragments. Lys\(^13\) can be cleaved by carboxypeptidase (Leslie, F. M., 1982) whereas the Arg\(^7\)-Ile\(^8\) peptide bond can be cleaved by enkephalinase (Shaw et al. 1982). A synaptic endopeptidase converts both Dyn A-(1-17) and Dyn A-(1-13) to Dyn A-(1-8) (Benuck et al., 1984).
Fig. 2.9. Metabolism of Dynorphin A-(1-13)
The resistance to enzymatic degradation can be increased by several ways. For bioassays, the use of peptidase inhibitors like thiorphan (an enkephalinase inhibitor), bestatin (an aminopeptidase inhibitor) and kelatorphan (which inhibits enkephalinase, amino-peptidase and diaminopeptidase) can reduce degradation considerably. Also lowering the temperature for incubations in radioligand binding assays can decrease degradation. Chemical modifications like substituting Gly\(^2\) by D-amino acids (e.g. D-Ala), or by converting the COOH terminal to an amide are recommended from a drug design point of view. Conformational restriction can also increase resistance to proteolysis and can be used in the design of metabolically stable and orally active compounds (Pals et al., 1986; Thaisrivongs et al., 1987).

2.2.5. Structure-Activity Relationships of Dyn A Analogues

The three-dimensional shape assumed by a peptide while interacting with its receptor is thought to determine its biological activity. Therefore information about the structural features that have a direct effect on biological activity is very important for understanding receptor structure and receptor-ligand interaction.

2.2.5.1. Modifications in the "Message" Sequence

The tetrapeptide N-terminal "message" sequence of dynorphin A has been the target of modification studies by several research groups. An alanine scan of each of the
residues in the tetrapeptide “message” sequence has revealed that the Tyr and Phe are very important for opioid activity and that substitutions in these positions cause substantial decreases in opioid activity and binding affinity (Turcotte et al., 1984). It is thought that the aromatic moiety of Tyr resembles the aromatic structure in the morphinan opioids. Phe also has been shown to be essential for opioid activity (Chavkin and Goldstein, 1981). Therefore modification in Gly-Gly sequence is often the target of modification in the “message” sequence studies. A systematic study of replacement of Gly in Dyn A-(1-13)NH₂ by various L-amino acids (e.g. Lys, Asn, Met and Ser) resulted in a decrease in opioid activity in the GPI, while retaining some κ-receptor selectivity. D-Amino acid substitution enhanced opioid activity, but the selectivity switched to μ-receptors (Story et al., 1992). However, substitution of Gly by D-Ala reduced opioid activity and also κ-receptor selectivity (Chavkin et al., 1981). Assuming that Gly-Gly acts as a spacer between Tyr and Phe, cis- and trans-aminocyclohexanecarboxylic acid were substituted in this region of Dyn A (Snyder et al., 1993). These analogues exhibited κ-receptor selectivity, but they proved to be quite weak agonists. The phenolic OH was shown to be important for agonist activity by Hruby and coworkers by replacement of Tyr by Phe (Kawasaki et al., 1993). Some other modifications at the N-terminal are the substitution of Phe by Phe(NO₂) (Schiller et al., 1982) and by Trp (Schiller, 1983), which reduced opioid activity in the GPI considerably. Also the N-terminal N,N-diallyl analogues were reported to be weak κ-receptor antagonists (Gairin et al., 1988). N-Substituted Monoalkyl derivatives exhibited better affinity than the N,N-diallyl substituted derivative and marked κ-receptor affinity (Choi et al., 1992). It could be the
effective relative orientation of two aromatic moieties, Tyr and Phe which contributes to changes in the opioid activity and receptor selectivity.

2.2.5.2. Modifications in the “Address” Sequence

Modifications have also been made to several residues in the C-terminal “address” sequence. The basic residues Arg and Lys have been shown to be important for opioid activity and they appear to contribute to κ-receptor selectivity (Goldstein 1981). Shortening Dyn A-(1-17) to Dyn A-(1-13) increased κ-receptor selectivity (Goldstein 1983). Substitution of Ile by D-Ile or D-Ala was found to increase κ-receptor selectivity slightly (Lemaire et al., 1986). Stereoisomeric replacement of Pro by D-Pro in Dyn A (1-13) (Lemaire et al., 1986) and in Dyn A-(1-11) (Gairin et al., 1985) increased kappa receptor selectivity. Dyn A-(1-13) or Dyn A-(1-11) are often used as the prototypes for further modifications to enhance κ-receptor selectivity.

Cyclization can be used to induce conformational constraint. [D-Cys-D/L-Cys]Dyn A-(1-13) show some κ-receptor selectivity (Kawasaki, et al., 1990). When further modification of D-Cys was done it improved binding affinity greatly, with modest κ-receptor selectivity (Kawasaki, et al., 1993). Other cyclic analogues [Orn-Asp] [Orn-Asp] and [Orn-Asp] show μ-receptor selectivity. Also cyclic analogues containing disulfide linkages between Cys/D-Cys/Pen (penicillamine)/D-Pen in positions 5 and 9, 10, 11 or 13 were examined by Hruby and coworkers and these analogues were
shown to have high κ and μ-receptor binding affinity, but very weak opioid activity (Meyer et al., 1994).

2.3. Solid Phase Synthesis

The technique of solid phase peptide synthesis (SPPS) (Fig. 2.10) introduced by R. B. Merrifield (Merrifield, 1963) opened up a new era in peptide synthesis. It eliminated the tedious work up and other problems encountered with solution synthesis, especially during the synthesis of long peptide chains. In solid phase synthesis, the growing peptide chain is attached to an insoluble polymeric support (see section 2.3.5), which makes it easier to remove the unreacted reagents and by-products by washing. The amino acids used have the N-terminus and certain side chains protected to avoid any side reactions during the coupling procedure. A “temporary” amine protecting group is removed quantitatively at each step to liberate the Nα-amine on the peptide resin. This is then followed by “coupling” of the next amino acid with its carboxyl group suitably activated. With repetitive cycles of deprotection and coupling, the peptide chain is built up one residue at a time. Then the linker to the support is cleaved to give the desired peptide. The protective functionalities on the side chain groups are also cleaved during this step.

There have been many recent developments in SPPS including new efficient coupling agents, different side chain protecting groups, different polymeric supports (resins), new linkers and new synthetic strategies.
Fig 2.10. The General Scheme of Solid Phase Peptide Synthesis
2.3.1. Temporary N<sub>a</sub>-Amine Protection

A temporary protecting group for the N<sub>a</sub>-amine of amino acid to be coupled is a basic requirement in SPPS (Barany and Merrifield, 1979). Depending on the N<sub>a</sub>-amine protection, there are two main synthetic strategies, based on the Boc (t-butyl-oxycarbonyl) and Fmoc (9-fluorenylmethoxycarbonyl) groups, which are currently used for SPPS (Fig. 2.11).

2.3.1.1. Boc Protection

The Boc group for N<sub>a</sub>-amine protection was first introduced in 1957 (McKay and Albertson, 1957). Boc is an acid-labile group and can be removed using 50% TFA (trifluoroacetic acid). The N<sub>a</sub>-amine is then neutralized, usually with a tertiary amine, followed by coupling of the next amino acid. The repetitive cycles of deprotection/coupling are performed until the desired peptide chain is formed. The side chain protecting groups used (usually of the benzyl type) must be stable towards the action of TFA, and usually are removed using a stronger acid like HF at the end of the synthesis. Also cleavage of the peptide from resin requires strong acid, i.e. HF or HBr in TFA.

The disadvantages of Boc protection are the repeated exposure to acidic media, which can result in the loss of peptide from the resin, and the use of a hazardous strong acid such as HF, which can lead to unwanted side reactions.
2.3.1.2. Fmoc Protection

The Fmoc protecting group was introduced by Carpino in 1972 (Carpino and Han, 1972). It has the advantage of providing an orthogonal protection strategy for SPPS. This is because Fmoc group is base-labile and can be removed by secondary amines such as piperidine or morpholine. This allows the use of acid-labile side chain protecting groups (usually the t-butyl type) which can be removed under mild acidic conditions. Usually TFA is used for final deprotection. Scavengers must be added to TFA to trap the reactive carbocation intermediates formed during acidolytic cleavage.

![Boc Protecting Group](image1)

![Fmoc Protecting Group](image2)

Fig. 2.11. The Nα-Amine Protecting Groups in SPPS
2.3.2. Side Chain Protecting Groups

Certain side chains of amino acids used in solid phase synthesis should be suitably protected to avoid potential side reactions during the synthesis or cleavage. Some amino acid residues (Cys, Asp, Glu, Lys) generally require the side chain protection, whereas it is recommended for other amino acids, and some residues (e.g. aliphatic amino acids) do not need any protection at all. Commonly used protecting groups for Boc and Fmoc chemistry (see also Fig. 2.12) are listed below.

Table 2.1. Protecting Groups Used for Various Amino Acids in Boc and Fmoc Chemistry

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Side Chain</th>
<th>Boc Chemistry</th>
<th>Fmoc Chemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp/Glu</td>
<td>-COOH</td>
<td>(OBzl) ester</td>
<td>(OtBu) ester/(OAl) ester</td>
</tr>
<tr>
<td>Ser/Thr</td>
<td>-OH</td>
<td>(Bzl) ether</td>
<td>(tBu) ether</td>
</tr>
<tr>
<td>Tyr</td>
<td>-OH</td>
<td>(Bzl) ether</td>
<td>(tBu) ether</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,6-C12Bz1/2-BrZ</td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>-NH2</td>
<td>2-ClZ/Fmoc/Aloc</td>
<td>Boc/Aloc</td>
</tr>
<tr>
<td>Arg</td>
<td>Guanidino</td>
<td>Tos/Mtr</td>
<td>Mtr/Pmc</td>
</tr>
<tr>
<td>Trp</td>
<td>Indole</td>
<td>Formyl (CHO)</td>
<td>Boc (optional)</td>
</tr>
<tr>
<td>Cys</td>
<td>-SH</td>
<td>4-MeBzl/Acm</td>
<td>Trt/Tmob/Acm</td>
</tr>
<tr>
<td>His</td>
<td>Immidazole</td>
<td>Tos/Dnp</td>
<td>Boc/Trt</td>
</tr>
<tr>
<td>Asn/Gln</td>
<td>-NH2</td>
<td>Xan (optional)</td>
<td>Trt/Tmob</td>
</tr>
</tbody>
</table>
Sometimes rearrangements take place even with protected amino acid residues, depending upon the sequence of the peptide, coupling and cleavage conditions used, type of resin used, etc. Research has provided some special side chain protecting groups to minimize these side reactions. The benzyl (Bzl) protecting group used in Boc chemistry for protection of the phenolic group of Tyr can migrate to the 3-position of the ring under strong acidic conditions (Erickson and Merrifield, 1973). The use of the 2,6-dichlorobenzyl (2,6-Cl₂Bzl) (Erickson and Merrifield, 1973) or the 2-bromobenzyl (2-BrZ) group (Yamashiro and Li, 1973) has been found to reduce this side reaction. Allyloxycarbonyl (Aloc) group provides orthogonal side-chain protection for Lys in both Boc and Fmoc chemistry (Lyttle and Hudson, 1992.). The Pmc group is preferred to Mtr for protection of Arg in Fmoc chemistry, since the deprotection of the Mtr group may require longer treatment (2-8 hours) with TFA, whereas Pmc can be readily removed (2 h) with TFA.
Fig. 2.12. Side Chain Protecting Groups in Fmoc Chemistry
2.3.3. Coupling Reactions of Protected Amino Acids

A variety of techniques are known to activate a carboxyl group which can then be coupled to the amino group of the adjacent amino acid residue. Common mechanisms for couplings in the solid phase peptide synthesis include active esters and the use of a variety of coupling reagents. (Fig. 2.13).

2.3.3.1. Active Esters

An early active ester used to couple amino acids was the p-nitrophenyl ester (ONp) (Bodanszky, et al., 1973, Bodansky et al., 1980) which was followed by N-hydroxysuccinimide (OSu) esters of Fmoc amino acids (Fields et al., 1973). OSu esters are not recommended for general use, since there is the potential risk of reaction of a succinimide carbonyl with the amine component (Gross and Bilk, 1968). HOBt (1-hydroxybenzotriazole) esters of Fmoc as well as Boc amino acids are formed rapidly with carbodiimides and are highly reactive (Fields et al., 1989). Pentafluorophenyl (OPfp) esters are also useful (Atherton et al., 1988). ODhbt (3-hydroxy-2,3-dihydro-4-oxobenzotriazine) esters are known to lower the racemization levels (Konig and Geiger, 1970). Fmoc amino acid esters of Hpp (1-(4-nitrophenyl)-2-pyrazolin-5-one), Pnp (3-phenyl-1-(4-nitrophenyl)-2-pyrazolin-5-one) and Npp (3-methyl-1-(4-nitrophenyl)-2-pyrazolin-5-one) are among the new agents in this field which react efficiently (Johnson et al., 1992, Hudson, 1990).
Fig 2.13. Active Esters of Fmoc/Boc Amino Acids

2.3.3.2. Coupling Reagents

The introduction of the first coupling agent DCC (N,N'-dicyclohexylcarbodiimide) (Merrifield, 1963) was followed by the introduction of DIC (N,N'-diisopropylcarbodiimide) (Sarantakis et al., 1976). This was advantageous for SPPS, since DCC gives insoluble DCU (dicyclohexylurea), whereas DIC gives a soluble urea by-product. Also the addition of HOBt was found to suppress racemization and N-acylurea formation (Mojsov et al., 1980, Konig and Geiger, 1970). Since then DIC with HOBt as an additive (Fig. 2.14) has become a preferred protocol for SPPS which accelerates the coupling rate and reduces racemization. HOAt (1-hydroxy-7-azabenzotriazole), an analogue of HOBt, has been recently introduced by Carpino and gives better results (see Chap 4) (Carpino, 1993).

Several new promising coupling agents have been recently introduced one after the other (Fig. 2.15). The phosphonium reagent BOP (1-benzo triazolyloxy-trisdimethylaminophosphonium hexafluorophosphate) is an efficient coupling agent (Castro, et al., 1975) (Fig. 2.16, proposed mechanism), but has the drawback of the formation of the carcinogenic byproduct HMPA. The phosphonium coupling agents PyBOP (benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate) (Coste et al., 1990) and PyBroP (bromotripyrrolidinophosphonium hexafluoride) (Coste et al., 1991) increase the efficiency of coupling in the case of hindered amino acids (Chap 4).

The new uronium reagents HBTU and TBTU (2-(1H-benzotriazole-1yl-1,1,3,3-tetramethyluronium hexafluorophosphate and tetrafluoroborate, respectively) (Knorr et al.,
1989) have been found to be useful. HATU (recent x-ray structure indicated in Fig. 2.15 (a) (Abdelmoty et al., 1994)) and HOAt (Carpino, 1993) HAPyU (O-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(tetramethylene)uronium hexafluorophosphate) (Ehrlich et al., 1993) are recent additions which appear quite promising.

![Coupling Mechanism of DIC in the Presence of HOBT](image)

**Fig. 2.14. Coupling Mechanism of DIC in the Presence of HOBT**

Fig. 2.15.(a) Coupling Reagents Used in SPPS
2.3.4. Monitoring Techniques

It is essential to confirm the completion of the coupling reaction prior to proceeding to the next step in order to avoid deletion sequences. The most common qualitative monitoring method used is the ninhydrin test for free primary \( \text{N}^\text{a} \) amino groups (Kaiser et al., 1970). This colorimetric test is performed on a small sample and is quick, easy, sensitive and reliable. A blue color indicates presence of the \( \text{N}^\text{a} \) amino group (except for Pro or other secondary amines), whereas the absence of blue coloration indicates the coupling is complete. The ninhydrin test can be also performed quantitatively. For secondary amines like Proline and other unusual amino acids, the isatin test (Kaiser et al., 1980) or chlroranil color tests (Christensen, 1979) can be performed to ensure complete coupling.
Fig. 2.16. Coupling Mechanism of BOP Reagent
2.3.5. The Solid Support and Linkages

The polymeric support and the linkage by which the C-terminal amino acid is anchored to it are very important factors in SPPS. Dry polystyrene beads, which usually have diameters of about 50 μm, can swell 3-6 times in volume with solvents routinely used in SPPS, namely DCM (dichloromethane) and DCM/DMA (N,N-dimethylacetamide) (Sarin et al., 1980). Several polymeric supports have been developed. The polyamide resin (commonly known as Pepsyn) (Arshady et al., 1981) is another commonly used resin. A new type of solid support, PEG-PS graft resin (polyethylene glycol polystyrene resin) (Bayer et al., 1990; Barany, et al., 1992), has polyethylene glycol linkages between a polystyrene (PS) gel bead and the handle or attachment point of the synthetic peptide. The “handle” is attached on one end to the functionalized resin and on the other end has a readily cleavable linkage to the peptide (Mitchell et al., 1978).

2.3.5.1. Resin Linkages Used in Boc Chemistry

Initially the Merrifield resin with a benzyl ester linkage, which can be cleaved by HF or HBr in TFA to produce a peptide acid, was common in Boc-chemistry. However it has a serious problem of loss of peptide from the resin when the N\textsuperscript{α} group is repetitively cleaved by TFA. Therefore a new handle PAM (4-hydroxymethylphenylacetamidomethyl) was introduced (Mitchell et al., 1978) (Fig. 2.16). The PAM resin has increased stability of the peptide resin linkage to acidolysis. The cleavage of the peptide-resin link-

age by HF and NH₃ can give a peptide acid and amide, respectively. The MBHA (4-methylbenzhydrylamine) resin (Matsuda and Stewart, 1981) has increased acid lability over BHA (benzhydrylamine) resin and is commonly used in Boc chemistry to produce peptide amides.

![Merrifield Resin](image)

Merrifield Resin

![Pam Resin](image)

Pam Resin

![MBHA Resin](image)

MBHA Resin

Fig. 2.17. Resins Used in Boc Chemistry
2.3.5.2. Resin Linkages Used in Fmoc Chemistry

Peptide acids were initially prepared using p-alkoxybenzyl resin and p-hydroxymethylphenoxy (HMP) linker. Then the PAC® resin with 4-hydroxymethylphenoxyacetic acid linker was also introduced to produce peptide acids. In order to increase acid lability, -OCH₃ group / groups can be inserted (Sheppard and Williams, 1982; Rink and Ernst, 1991). Peptide amides are usually prepared with the PAL (Peptide Amide Linker) resin (Albericio and Barany, 1987). This is an MBHA resin with a 5-(4-aminomethyl-3,5-dimethoxyphenoxy) valeric acid linker.

For Synthesis of Peptide Acids

![4-alkoxybenzyl alcohol resin](image1)

4-alkoxybenzyl alcohol resin

![4-hydroxymethylphenoxyacetic acid](image2)

4-hydroxymethylphenoxyacetic acid

![3-(4-hydroxymethylphenoxy)propionic acid](image3)

3-(4-hydroxymethylphenoxy)propionic acid

Fig. 2.18. Resins Used in Fmoc Chemistry
Fig. 2.18. (continued)
CHAPTER 3

SYNTHESIS AND OPIOID ACTIVITY OF DYNORPHIN A-(1-11) ANALOGUES
WITH A CONFORMATIONAL CONSTRAINT IN THE
"MESSAGE" SEQUENCE

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3.1. Abstract

The main goal of this research was to identify analogues of the peptide dynorphin A (Dyn A) with antagonist activity. Since the N-terminal tetrapeptide “message” sequence (Tyr-Gly-Gly-Phe) of dynorphin A (Dyn A) is believed to be important for opioid activity, we prepared a series of [D-Pro$^{10}$]Dyn A-(1-11)OH analogues with modifications in the message sequence. A tetrapeptide Tyr-Tic-Phe-Phe-OH (TIPP) as well as the tripeptide Tyr-Tic-Phe-OH (TIP), which contain only aromatic residues, were synthesized by Schiller and coworkers and reported to be selective δ-receptor antagonists. (Schiller, et al., 1992a; Schiller, 1993). Therefore in our attempt to develop antagonists based upon the structure of Dyn A, we chose to attach TIPP and TIP to the C-terminal sequence of Dyn A. We further modified the TIPP sequence by replacing Tic$^2$ (1,2,3,4-tetrahydroisoquinolinecarboxylic acid) by structurally related residues Pip (pipelicolic acid) and NMePhe (N-methylphenylalanine). Further modification of the TIPP sequence was achieved by substitution of Phe$^3$ by D-Phe and Gly. We also synthesized control compounds for each of these peptides with Phe in position 2 in order to evaluate and compare the opioid activity with the parent peptide in each series. All of these peptides were synthesized by solid phase peptide synthesis on a hydroxymethylphenoxyacetic acid resin (PAC) using Fmoc-protected amino acids. We used the standard coupling protocol for Phe$^2$ peptides, but we anticipated difficulty in coupling for the peptides containing hindered amino acids and the possibility of side product formation due to incomplete couplings. Therefore the synthetic strategy was modified for the synthesis of these
peptides and the last two residues were coupled manually using a more efficient coupling agent, PyBOP (Coste et al., 1990; Hoag-Jansen et al., 1991). The peptides were purified by reverse phase HPLC.

Opioid receptor affinities were evaluated in radioligand binding assays using cloned opioid receptors and opioid activity was evaluated in the GPI (guinea pig ileum). All the analogues containing hindered amino acids (Tic/Pip/NMePhe) in position 2 showed higher κ-receptor affinity ($K_i = 13-35$ nM) than analogues containing Phe$^2$ ($K_i = 21-217$ nM) in the radioligand binding assays. Preliminary GPI experiments indicate that all of the analogues except the Phe$^3$ analogues examined to date possessed weak agonist activity ($IC_{50} > 500$ nM), with agonist activity slightly greater for the substituted analogues (containing Tic/Pip/NMePhe) than the Phe$^2$ analogues. The analogues with Gly$^3$ (group C) showed no agonism even at 3 μM, which might be indicative that the hindered residue should be followed by an aromatic residue (Phe$^3$) to demonstrate opioid agonist activity. The [Tic$^2$,Gly$^3$] analogue is being examined for possible antagonist activity. Further testing to confirm these results are in progress. These data suggests that this approach may result in interesting lead compounds for the development of κ antagonists.

Keywords: dynorphin A analogues, message sequence, message-address concept
3.2. Abbreviations

Abbreviations used for amino acids are according to the rules specified by the IUPAC-IUB Joint Commission of Biochemical Nomenclature in Eur. J. Biochem. 1984, 138, 9-37. All amino acids are in the L configuration unless otherwise specified.

Additional abbreviations used are BOP, (benzotriazol-1-yl-oxy-tri(dimethylamino)phosphonium hexafluorophosphate; CHO (Chinese hamster ovary); DAMGO, Tyr-D-Ala-Gly-MePhe-NH(CH₂)₂OH; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DIPEA, N,N'-diisopropylethylamine; DMA, N,N-dimethylacetamide; DPDPE, cyclo[D-Pen²,D-Pen⁵]enkephalin; EDT, ethanedithiol; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenlymethoxycarbonyl; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; HOAt, 1-hydroxy-7-azabenzotriazole; HATU, O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethylenuronium hexafluorophosphate; NMePhe, N⁴-methylphenylalanine; Pip, pipecolic acid; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfanyl; PyBOP, benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.
3.3. Introduction and Rationale

The conformation assumed by a peptide during its interaction with its receptor is an important factor in determining the biological activity of the peptide. A conformational constraint which restricts the freedom of a peptide can significantly alter a peptide’s biological activity, including converting a peptide from an agonist to an antagonist (Pena et al., 1974, Turk et al., 1976). Conformational restriction can also impart other properties to peptides, such as increased resistance to proteolysis, and can be used in the design of metabolically stable and orally active peptides (Thaisrivongs et al., 1987). Replacing the key sequences in a peptide by rigid/sterically bulky amino acid residues is one common approach for conformational constraint (Schiller, 1991).

Dynorphin A (Dyn A), isolated from porcine pituitary (Cox et al., 1975; Goldstein, et al., 1979), is postulated to be an endogenous ligand for κ opioid receptors (Chavkin, et al, 1981). It has been proposed that Dyn A has two distinct regions, the N-terminal tetrapeptide “message” sequence (Tyr-Gly-Gly-Phe) and the remaining C-terminal “address” sequence (Chavkin et al., 1982; Schwyzer, et al., 1977) (Fig. 2.7). The “message” sequence of Dyn A has been shown to be important for opioid activity (Chavkin et al., 1982), and in particular Tyr¹ and Phe⁴ are believed to be important for opioid activity (Chavkin and Goldstein, 1981). In a series of Dyn A-(1-13) analogues involving systematic substitution of each amino acid by Ala, the analogues with Tyr¹ and Phe⁴ substituted by Ala showed large decreases in affinity and potency in the GPI, sup-
porting this conclusion (Turcotte et al., 1984). Therefore modifications in the Gly2-Gly3 sequence is the target of our modifications in the “message” sequence.

A number of Dyn A analogues have been synthesized, either by replacing amino acid residues in the “message” sequence or by modifying the N-terminus, in attempts to develop κ-receptor selective antagonists. The analogue [Ala2,Trp4]Dyn A-(1-13) was reported to have κ-antagonist properties (Lemaire et al., 1986a). Substitution of D-Trp2 in position 2 and also positions 3, 4 or 5 could impart weak antagonist activity to Dyn A (Gairin, et al., 1986; Lemaire et al., 1986b). [Aib2]DynA-(1-13) (Aib = α-aminoisobutyric acid) showed loss of κ-receptor selectivity and a large decrease in opioid activity, but N,N-diallylated peptides with Aib2 exhibited weak antagonist property (Gairin et al., 1988; Lemaire et al., 1990). N-Substituted monoalkyl derivatives exhibited better affinity than N,N-diallyl substituted derivatives (Choi et al., 1992).

Other analogues with modifications in positions 2 and 3 have been examined for their effect on opioid receptor selectivity and opioid activity. Analogues involving substitution of Gly2 by various L-amino acids in Dyn A-(1-13)NH2 retained some κ-receptor selectivity, but their opioid activity decreased, whereas substitution by D-amino acids increased opioid potency in the GPI, but the receptor selectivity profile shifted to μ-receptors (Story et al., 1992). Replacing Gly2-Gly3 by cis and trans-4-aminocyclohexanecarboxylic acid (ACCA), [cis-ACCA2-3] and [trans-ACCA2-3] DynA-(1-13)NH2 analogues were prepared and shown to possess slightly better κ-receptor selectivity but lower opioid activity in GPI than Dyn A-(1-13) amide (Snyder et al., 1993). These results indicate that the Gly2-Gly3 bond is not critical for κ opioid receptor affinity.
Recently Schiller and coworkers synthesized the tetrapeptide TIPP (Tyr-Tic-Phe-Phe-OH) and tripeptide TIP (Tyr-Tic-Phe-OH) which consist entirely of aromatic residues (Schiller et al., 1992a; Schiller, 1993). Introduction of Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) was found to produce a significant effect on receptor selectivity and opioid activity of the peptides. Both TIPP (H-Tyr-Tic-Phe-Phe-OH) and TIP (H-Tyr-Tic-Phe-OH) showed very high δ-receptor affinity, and high potency as δ antagonists (Schiller et al., 1993).

Therefore in our attempt to develop κ-receptor antagonist compounds, we substituted the message sequence of Dyn A (Tyr-Gly-Gly-Phe) with the tetrapeptide fragment of TIPP or the tripeptide TIP. [D-Pro\textsuperscript{10}]Dyn A-(1-11) was chosen as the parent peptide since it has been reported to have enhanced κ-receptor selectivity (Gairin et al., 1985; Gairin, et al., 1988). Pip (pipecolic acid) is a conformationally restricted amino acid and NMePhe (N-methylphenylalanine) is a hindered residue (Fig. 3.1), both of which closely resemble Tic in structure. Therefore we also synthesized [TIPP,D-Pro\textsuperscript{10}]Dyn A-(1-11) analogues in which Tic\textsuperscript{2} was substituted by the hindered amino acid residues Pip\textsuperscript{2} and NMePhe\textsuperscript{2}. In order to determine the importance of the aromatic residue Phe\textsuperscript{3} in TIPP on opioid activity, we substituted DPhe\textsuperscript{3} or Gly\textsuperscript{3} for Phe\textsuperscript{3} and synthesized several analogues of [TIPP,D-Pro\textsuperscript{10}]Dyn A-(1-11). Since a change in stereochemistry can markedly alter biological activity DPhe\textsuperscript{3} was substituted for Phe\textsuperscript{3}. We also substituted Phe\textsuperscript{3} with Gly\textsuperscript{3} to determine whether an aromatic residue is needed in position 3 of the TIPP sequence. Truncating the sequence has been one method for developing shorter receptor selective opioid peptide analogues (Schiller, 1991). By using the TIP (tripeptide) fragment as the
message sequence we prepared an analogue with a truncated “message” sequence (referred to as desGly\(^3\)). For each of the peptides with a hindered amino acid in position 2, we synthesized a control peptide with Phe in position 2.

All of these modifications could alter the conformation of Dyn A, particularly the relative orientation of aromatic residues in positions 1 and 4 in the “message sequence”, which could thereby alter opioid activity. We also synthesized initial analogues by substituting position 2 by D-amino acids (Appendix).

All of the peptides were prepared by solid phase synthesis on hydroxymethylphenylacetic acid resin (PAC\(^\circledR\)) using the Fmoc synthetic strategy. However the synthetic strategy was modified for the last two couplings and PyBOP was used as the coupling agent for all peptides containing a hindered amino acid residue. The peptides were cleaved from the resin using reagent K (King et al., 1990). These peptides are being evaluated for opioid receptor affinity in radioligand binding assays and for opioid activity in the guinea pig ileum (GPI).
Fig. 3.1. Hindered Amino Acid Residues
3.4. Materials and Methods

3.4.1. Materials.

The reagents used in peptide synthesis were as follows: HOBt and all standard Fmoc-protected amino acids (Bachem California, Torrence, CA), Fmoc-Tic, Fmoc-Pip and Fmoc-NMePhe (Advanced ChemTech, Louisville, Ky), TFA (Pierce Chemical Co. Rockford, IL), thioanisole, ethanediithiol, DIC, and piperidine, (Aldrich, Milwaukee, WI); HPLC grade solvents, DMA, DCM and toluene (Aldrich / Burdick & Jackson Inc., Muskegon, MI); hydroxymethylphenoxyacetic acid resin (PAC® resin), DIPEA, HOAt and HATU (Millipore, Bedford, MA); PyBOP (NovaBiochem La Jolla, CA).

The peptides were synthesized using the Fmoc chemical protocol on a Biosearch 9500 automated peptide synthesizer (Novato, CA). Side-chain protecting groups used for the amino acids were tert-butoxycarbonyl (Boc) for Lys, 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for Arg and tert-butyl (tBu) for Tyr. Analytical HPLC was carried out for crude as well as pure samples on a Beckman Model 431A system consisting of two model 110B pumps, a model 421 controller, a model 201A injector, a model 163 detector and a Waters model 740 data module and Vyadac C18 reversed phase column (dimensions: 4.6 mm x 250 mm, particle size 10 µ and pore size 300 Å). Purification of the peptides was performed on a Rainin Dynamax gradient HPLC system. The elution was monitored at 280 nm using an ISCO UA5 absorbance detector or Shimadzu model 10 UVA detector. The solvents used for HPLC were solvent A, aqueous 0.1 % TFA, and solvent B, AcCN containing 0.1 % TFA. The desired peptides were characterized by
FAB-MS (fast atom bombardment mass spectrometry), amino acid analysis and analytical HPLC. FAB-MS spectra were obtained on a Kratos MS50RF spectrometer in the Department of Agricultural Chemistry at Oregon State University, Corvallis, OR. Individual products were characterized by subjecting the mixture of crude peptides to ion spray mass spectrometry performed on a PE SCIEX, API3, biomolecular mass analyzer, in the Department of Agricultural Chemistry, Oregon State University, Corvallis, OR. The ion spray voltage used was 5000 V and the oriface voltage was 80 V. Amino acid analysis was performed on a Beckman System 6300 amino acid analyzer at the Protein and Nucleic Acid Facility, Beckman Center, Stanford University Medical Center, Stanford, CA. The peptide samples were hydrolyzed using 6 N HCl prior to amino acid analysis.

3.4.2. Peptide Synthesis Using Fmoc-protected Amino Acids

The peptides were assembled on a hydroxymethylphenoxyacetic acid resin (PAC® resin) as described below. The Fmoc-Lys(Boc)-PAC resin (0.5 g, 0.36 mmol/g substitution) was washed with 10 mL of DCM / DMA (1/1, 3x) and then allowed to swell in DCM (20 mL) for about 30 min. Then deprotection of the Fmoc group was carried out for 3 min, then 7 min using piperidine/toluene/DMA (30/35/35, v/v) and the resin was then washed with DCM/DMA (1/1, 12x). Except for the hindered Fmoc-amino acids* (see below), all of the remaining Fmoc-amino acids were coupled to the growing peptide chain for 2 h using a 4-fold excess of the amino acids in DMA and equimolar amounts of DIC (0.4 M in DCM) and HOBt. Qualitative ninhydrin analysis was performed on small
samples of resin after each coupling to determine whether reactions were complete (Kaiser et al., 1970). In the case of secondary amine couplings (Fmoc-Tic/Fmoc-Pip/Fmoc-NMePhe) the chloranil color test was performed (Christensen, 1979) to determine when couplings were complete. Fmoc deprotection was performed in two steps, and the next amino acid was coupled as described above.

*In the case of the peptides containing a hindered amino acid, the standard coupling procedure was modified (Fig. 3.2) and the couplings of the last two residues (after the coupling of Fmoc-Phe³) were performed manually. The Fmoc-protected hindered amino acids (Fmoc-Tic/Fmoc-Pip/Fmoc-NMePhe) were used in a two-fold excess and Fmoc-Tyr(tBu) was used in a four-fold excess. The base wash steps were manually performed (2x) for extended times (~15 min each) to ensure that the deblocking of the Fmoc group was complete before coupling of Fmoc-Tyr(tBu) to the hindered amino acid. Also Fmoc-Tyr(tBu) was double coupled in the case of all of these peptides. The Fmoc-hindered amino acid or Fmoc-Tyr(tBu) and HOBT (1:1) were mixed in DMA (0.4 M solution) and were added to the resin after the base washes. PyBOP (1 equivalent) with DIPEA (3 equivalent) were then added manually to the reaction vessel, and the contents were mixed well under nitrogen with precautions taken to maintain the pH around 7-8 (extra DIPEA was added three times to maintain the pH). The completion of the coupling was checked by the ninhydrin or chloranil test and coupling times were noted (2 h for Fmoc-Tic and (3+1) h for Fmoc-Tyr(tBu)). The resin was then washed with DCM/DMA (1/1, 5x).
After completion of the peptide assembly and removal of the Fmoc group from the N-terminal residue with piperidine/toluene/DMA, the resin was washed successively with DCM/DMA (1/1, 3x) and MeOH (5x, 15 min). The resin was then collected and dried overnight in vacuo.

3.4.3. Cleavage of the Peptide from the Support

The dried protected resin was treated with 5 mL of Reagent K (82.5% TFA, 5% phenol, 5% thioanisole and 2.5% ethanedithiol) (King et al., 1990). The reaction flask was flushed with nitrogen and the reaction then continued at room temperature for about 2 hours. In the case of peptides containing a hindered residue, the deprotection time was extended to 3 h, since crude peptides were observed to contain about 8% of a peptide containing tyrosine with the side chain protecting t-butyl group intact when the shorter deprotection time was used. The TFA solution was filtered from the resin, the resin was washed with 1 mL of TFA, and then the TFA solution was concentrated to about half of its original volume by evaporation with nitrogen for about 20-30 min. The concentrated solution was then diluted with 40 mL of 10% AcOH (20% AcOH in peptides containing Tic in order to avoid emulsion formation) and then extracted with anhydrous Et₂O (3x). The aqueous layer was then lyophilized to give the crude peptide in each case.
Fig. 3.2. Synthetic Strategy for Peptides Containing Hindered Amino Acids
3.4.4. Purification of the Peptides

The crude peptides were purified by preparative reversed phase HPLC at a flow rate of 20 mL/min using one of three linear gradients: A) 0 % - 75 % B over 50 min (for peptides with standard amino acids in the sequence); B) 20 % - 75 % B over 40 min and then to 80 % B in the next 10 min; or C) 20 % - 60 % B over 40 min. and then to 80 % B in 10 min (B and C were used for the peptides containing hindered amino acid residues). All of the peptides were obtained in pure form after one chromatographic purification except for [Tic²,desGly³], which required a second purification. The peak fractions were analyzed by isocratic analytical HPLC and pure fractions combined and lyophilized. The resulting pure peptides were characterized by FAB-MS, amino acid analysis and gradient analytical HPLC (Tables 3.1 and 3.2).
Table 3.1 Characterization of [TIPP,D-Pro^{10}]Dyn A-(1-11) analogues

General compound structure: [(X^{2},Y^{3}),D-Pro^{10}]Dyn A-(1-11)

\[
\text{Tyr}^{1}\cdot \text{X}^{2}\cdot \text{Y}^{3}\cdot \text{Phe}^{4}\cdot \text{Leu}^{5}\cdot \text{Arg}^{6}\cdot \text{Arg}^{7}\cdot \text{Ile}^{8}\cdot \text{Arg}^{9}\cdot \text{D-Pro}^{10}\cdot \text{Lys}^{11}\cdot \text{OH}
\]

<table>
<thead>
<tr>
<th>[TIPP,D-Pro^{10}] Dyn A-(1-11)</th>
<th>Gradienta HPLC R_t</th>
<th>FAB-MS M+1 (calculated)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Phe^{2},Phe^{3}</td>
<td>27.2</td>
<td>1543 (1542.8)</td>
</tr>
<tr>
<td>2. Tic^{2},Phe^{3}</td>
<td>25.5</td>
<td>1555 (1554.9)</td>
</tr>
<tr>
<td>3. Pip^{2},Phe^{3}</td>
<td>24.1</td>
<td>1507 (1506.9)</td>
</tr>
<tr>
<td>4. N-MePhe^{2},Phe^{3}</td>
<td>24.05</td>
<td>1557 (1556.9)</td>
</tr>
<tr>
<td><strong>Group B</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Phe^{2},DPhe^{3}</td>
<td>27.5</td>
<td>1543 (1542.8)</td>
</tr>
<tr>
<td>6. Tic^{2},DPhe^{3}</td>
<td>25.7</td>
<td>1555 (1554.9)</td>
</tr>
<tr>
<td><strong>Group C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Phe^{2},desGly^{3}</td>
<td>26.0</td>
<td>1396 (1395.8)</td>
</tr>
<tr>
<td>8. Tic^{2},desGly^{3}</td>
<td>23.6</td>
<td>1408 (1408.1)</td>
</tr>
<tr>
<td><strong>Group D</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. Phe^{2},Gly^{3}</td>
<td>23.5</td>
<td>1453 (1452.8)</td>
</tr>
<tr>
<td>10. Tic^{2},Gly^{3}</td>
<td>24.1</td>
<td>1465 (1464.9)</td>
</tr>
</tbody>
</table>

*aConditions: Vydac C_{18} reverse phase column, 0% B to 75% B over 50 min with the flow rate 1.5 mL/min.
Table 3.2. Amino Acid Analysis of [TIPP,D-Pro\textsuperscript{10}]Dyn A-(1-11) Analogues\textsuperscript{a}

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Tyr(1)</th>
<th>X\textsuperscript{b}(1)</th>
<th>Gly(1)</th>
<th>Phe(2)</th>
<th>Leu(1)</th>
<th>Arg(3)</th>
<th>Ile(1)</th>
<th>DPro(1)</th>
<th>Lys(1)</th>
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<tbody>
<tr>
<td><strong>Group A</strong></td>
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<tr>
<td>1. Phe\textsuperscript{2},Phe\textsuperscript{3}</td>
<td>1.07</td>
<td>-</td>
<td>-</td>
<td>2.94(3)</td>
<td>1.05</td>
<td>2.90</td>
<td>0.95</td>
<td>1.06</td>
<td>1.04</td>
</tr>
<tr>
<td>2. Tic\textsuperscript{2},Phe\textsuperscript{3}</td>
<td>1.03</td>
<td>1.05</td>
<td>-</td>
<td>1.95</td>
<td>1.05</td>
<td>2.90</td>
<td>0.94</td>
<td>1.07</td>
<td>1.03</td>
</tr>
<tr>
<td>3. Pip\textsuperscript{2},Phe\textsuperscript{3}</td>
<td>0.96</td>
<td>1.00</td>
<td>-</td>
<td>2.15</td>
<td>1.00</td>
<td>2.96</td>
<td>0.93</td>
<td>1.01</td>
<td>1.00</td>
</tr>
<tr>
<td>4. N-MePhe\textsuperscript{2},Phe\textsuperscript{3}</td>
<td>0.90</td>
<td>1.14</td>
<td>-</td>
<td>1.98</td>
<td>1.01</td>
<td>2.99</td>
<td>0.95</td>
<td>1.03</td>
<td>1.00</td>
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<tr>
<td><strong>Group B</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5. Phe\textsuperscript{2},DPhe\textsuperscript{3}</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>3.03(3)</td>
<td>1.02</td>
<td>2.96</td>
<td>0.93</td>
<td>1.04</td>
<td>1.01</td>
</tr>
<tr>
<td>6. Tic\textsuperscript{2},DPhe\textsuperscript{3}</td>
<td>0.75</td>
<td>0.95</td>
<td>-</td>
<td>1.97</td>
<td>1.01</td>
<td>3.10</td>
<td>0.99</td>
<td>1.10</td>
<td>1.11</td>
</tr>
<tr>
<td><strong>Group C</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7. Phe\textsuperscript{2},desGly\textsuperscript{3}</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>2.02</td>
<td>1.03</td>
<td>2.96</td>
<td>0.95</td>
<td>1.04</td>
<td>1.01</td>
</tr>
<tr>
<td>8. Tic\textsuperscript{2},desGly\textsuperscript{3}</td>
<td>1.02</td>
<td>1.06</td>
<td>-</td>
<td>0.95(1)</td>
<td>1.04</td>
<td>2.90</td>
<td>0.93</td>
<td>1.06</td>
<td>1.06</td>
</tr>
<tr>
<td><strong>Group D</strong></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>9. Phe\textsuperscript{2},Gly\textsuperscript{3}</td>
<td>0.99</td>
<td>-</td>
<td>1.00</td>
<td>2.00</td>
<td>1.02</td>
<td>3.00</td>
<td>0.95</td>
<td>1.03</td>
<td>1.01</td>
</tr>
<tr>
<td>10. Tic\textsuperscript{2},Gly\textsuperscript{3}</td>
<td>0.95</td>
<td>1.01</td>
<td>1.03</td>
<td>0.99(1)</td>
<td>1.01</td>
<td>2.98</td>
<td>0.95</td>
<td>1.04</td>
<td>1.04</td>
</tr>
</tbody>
</table>

\textsuperscript{a} (Expected value in parenthesis) and \textsuperscript{b}X = Tic/ Pip/NMePhe}
3.5. Binding Assays for Opioid Receptor Binding

The binding affinity was investigated using cloned opioid receptors (κ, μ, δ respectively) expressed in CHO (Chinese hamster ovary) cells (Bunzow et al., 1995). The cells were harvested after 72 h, followed by transfection in 50 mM Tris buffer (pH=7.4 at 4°C) and centrifugation at 45,000 x g for 10 min at 4°C. Then the pellet was resuspended and recentrifuged twice as described above. The pellet was washed twice and resuspended in 50 mM Tris buffer to yield a protein concentration in the range of 30-60 µg/mL. The binding assays were performed at 22°C for 90 min in polypropylene tubes with [³H]diprenorphine, [³H]DAMGO and [³H]DPDPE for κ, μ and δ receptors, respectively. Bestatin, captopril, and L-leucyl-L-leucine were added in concentrations of 10, 30 and 50 µM. The tubes also contained Mg²⁺ (3 mM). The peptide aliquot (20 µL) was added to give final concentrations from 0.1 nM to 10 µM in a final volume of 2 mL. One mL of membrane was added to start the binding reaction. Unlabeled ligands (Dyn A (1-13)NH₂ for κ, DAMGO for μ, DPDPE for δ) (10 µM concentration) were used to determine nonspecific binding. Whatman GF/B fiber filters were soaked for 2 h in polyethyleneimine to reduce nonspecific binding to the filters. The reactions were terminated by rapid filtration using a Brandel M24-R cell harvester. The filter disks were placed in minivials along with Cytocint (4 mL) and allowed to elute for 6 h before counting in a Beckman LS6800 scintillation counter. The Cheng and Prusoff equation (Cheng and Prusoff, 1973) was used to calculate dissociation constants (Kᵢ) from the IC₅₀ values derived from competition analyses. Kᵢ values determined for tritiated diprenorphine, DAMGO and DPDPE
were 0.45, 0.76 and 7.63 nM respectively. The results of κ-receptor binding assays are presented in Table 3.3. The binding assays for μ and δ are in progress.

3.6. Guinea Pig Ileum Assays

The peptides are being evaluated for opioid activity and for antagonist activity against Dyn A-(1-13)NH₂ in the electrically stimulated muscle of the guinea pig ileum (GPI), as described elsewhere (Story et al., 1992). The only modification was addition of peptidase inhibitors, 100 nM bestatin (200 μL) and 3 nM thiorphan (200 μL) (McKnight et al., 1983), to the bath 6 min prior to the addition of the tested peptides. Agonist activity was calculated as percent inhibition of the twitch. At least three replications are performed using tissues from different animals and the mean IC₅₀ value calculated from cumulative dose-response curves. Dyn A-(1-13)NH₂ was used as a control in each tissue and the IC₅₀ value for it was determined; only test results from tissues with acceptable IC₅₀ values for Dyn A were used. In order to determine antagonist activity, the peptides to be tested were added to the bath in the presence of peptidase inhibitors 5 min before adding Dyn A-(1-13)NH₂. The concentration of peptide used was determined from cumulative dose-response curves. For antagonism testing, peptides doses were selected so that they caused less than 20 % agonism when administered alone. The results are presented in Table 3.4
3.7. Results

3.7.1. Peptide synthesis

The standard solid phase synthetic strategy was modified to minimize anticipated difficulties in the couplings involving the hindered amino acids (Fig. 3.2). The standard DIC/HOBt method was used for all standard amino acids, but the classical reagent DIC/HOBt gives poor results in couplings with hindered amino acids, even with long reaction times. Therefore PyBOP (Coste et al., 1990 Hoag-Jenson et al., 1991 and Ferrot et al., 1991), which is reported to be an efficient coupling agent which minimizes side reactions, was used for coupling of the hindered amino acids. Crude peptides were cleaved from the resin using reagent K (82.5% TFA/ 5%.phenol/ 5% H₂O/ 5% thioanisole/ 2.5% EDT) (King et al., 1990).

Although peptides with only standard amino acids yielded the desired peptide with minimal side products (Fig. 3.3a), the peptides with a hindered amino acid (Tic/Pip/NMePhe) in position 2 consistently contained considerable amounts of side products due to mainly incomplete couplings (Fig. 3.3b). We used PyBOP, an efficient coupling agent, for coupling of the last two residues and carefully monitored the reactions (maintaining pH ~ 8) to maximize the reaction rate. In spite of this, we observed similar undesired side products in all of these crude peptides. LC-MS (liquid chromatography combined with on-line mass spectrometry) was used to characterize the side products. The main products obtained from the synthesis of peptides containing a
hindered amino acids were a) the desired peptide, b) the [des-Tyr] peptide (due to incomplete coupling of Fmoc-Tyr(tBu) coupling), c) the [des-Tyr-des-X] peptide, and d) the [des-X] peptide (where X = Tic / Pip / NMePhe) (Fig. 3.3b). The base wash steps were performed for an extended time (~15 min) to ensure complete deprotection of Fmoc group before coupling of Fmoc-Tyr(tBu). Due to the side products, the percentage of desired peptides was low (~30%). Purity of the desired peptide and a high yield are very important factors in synthesis, so we conducted a comparative study of various coupling agents (PyBOP, PyBrop and HATU) and coupling additives (HOBt Vs HOAt) (see Chapter 4).
A) HPLC profile for [Phe², Phe³, D-Pro¹⁰]Dyn A-(1-11)

B) HPLC profile for the Dyn A analogues containing hindered amino acids (Tic/Pip/NMePhe)

Fig. 3.3. Representative Analytical HPLC Tracings of Crude Peptides Cleaved Using Reagent K
3.7.2. Pharmacological Testing

We are evaluating the binding affinities of the pure peptides by displacing \(^{3}\text{H}\)diprenorphine (\(\kappa\)), \(^{3}\text{H}\)DAMGO (\(\mu\)), and \(^{3}\text{H}\)DPDPE (\(\delta\)) from cloned opioid receptors expressed in CHO cells (Table 3.3). The opioid activity of these peptides is being evaluated using the electrically stimulated contraction of the guinea pig ileum (GPI) (Table 3.4).

3.7.2.1. Binding Affinity

The \(\kappa\)-receptor binding assays (Table 3.3) indicate that there is a marked increase in \(\kappa\)-receptor affinity (~4-fold) for most of the peptides containing a hindered amino acid residue in position 2 compared to the control compound containing Phe\(^{2}\) (Table 3.3) with two exceptions. All of the Tic\(^{2}\) peptides showed modest affinity for \(\kappa\)-receptors (\(K_{i} = 15-26\) nM), while their respective control compounds containing Phe\(^{2}\) generally exhibited significantly lower affinity (\(K_{i} = 71-217\) nM) except for the peptide \([\text{Phe}^{2},\text{DPhe}^{3}]\) (23 nM). For the peptides in group C (the des-Gly series) a higher increase in \(\kappa\)-receptor affinity (~8-9 X) was observed when Phe\(^{2}\) was substituted by Tic\(^{2}\). The other exception was the group B compounds (containing DPhe\(^{3}\)), which did not show much increase in affinity by substitution of Phe\(^{2}\) with Tic\(^{2}\). This indicates that position 2 in the message sequence of Dyn A plays a very important role in \(\kappa\) opioid receptor affinity and conforma-
tional restriction by Tic² substitution can enhance κ-receptor affinity. The peptides containing Pip or NMePhe in position 2 (Group A series) also showed an increase (4 X) in κ receptor affinity compared to the control compound. Pip (pipecolic acid), NMePhe and Tic possess some structural similarities, although there are key structural differences (Fig. 3.1). N-MePhe is a ring opened analogue of Tic, whereas removal of an aromatic ring from Tic gives the structure of Pip. Since all these peptides showed similar κ-receptor binding affinity, it appears that the aromatic residue in position 2 of TIPP sequence in these Dyn A analogues is not critical for κ-receptor affinity. The μ and δ receptor binding assays are in progress.

3.7.2.2. GPI Experiments

Initially all analogues were evaluated for agonist activity in GPI. Preliminary experiments for agonist testing of group A peptides indicated that the control compound [Phe²,Phe³] showed extremely weak agonist activity with doses of 10 μM causing less than 30 % inhibition. The extremely weak agonist activity for this compound is consistent with the weak binding affinity observed for κ-receptors. Most of the compounds with a modified message sequence showed weak agonist activity, with IC₅₀ values greater than 1 μM. Only the [Pip²,Phe³] analogue showed substantial agonist activity at doses less than 1 μM (IC₅₀ ~ 520 nM). The Gly³ analogues [Tic²,Gly³] and [Phe²,Gly³] analogues, however showed negligible agonist activity at doses up to 3 μM. The [Tic²,Gly³] analogue is currently being investigated for antagonist activity.
During the course of this investigation, Schiller and his coworkers reported the synthesis of [TIPP,D-Pro^{10}]Dyn A-(1-11)NH₂, which showed weak kappa receptor affinity (Kᵢ = 49.1 nM) and weak kappa antagonist activity (Kₑ = 279 nM) in the GPI (Schmidt, et al., 1994). The only difference from our analogue [TIPP,D-Pro^{10}]Dyn A-(1-11)OH, was the C-terminal functionality which is an amide rather than an acid as in our peptide.
Table 3.3. Opioid Receptor Binding Affinities

\[^{X^2,Y^3,D-Pro^{10}}\text{Dyn A-(1-11) Analogues } K_i (\text{nM})^a\]

<table>
<thead>
<tr>
<th>[^{X^2,Y^3,D-Pro^{10}}\text{Dyn A-(1-11) Analogue}</th>
<th>K_i (\text{nM})^{\text{[^3]H}diprenorphine}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dyn A-(1-13)NH₂</td>
<td>0.56 ± 0.17</td>
</tr>
<tr>
<td><strong>Group A</strong></td>
<td></td>
</tr>
<tr>
<td>1. [Phe²,Phe³]</td>
<td>80.7 ± 4.7</td>
</tr>
<tr>
<td>2. [Tic²,Phe³]</td>
<td>18.4 ± 1.5</td>
</tr>
<tr>
<td>3. [Pip²,Phe³]</td>
<td>13.8 ± 1.6</td>
</tr>
<tr>
<td>4. [N-MePhe²,Phe³]</td>
<td>15.9 ± 3.7</td>
</tr>
<tr>
<td><strong>Group B</strong></td>
<td></td>
</tr>
<tr>
<td>5. [Phe²,DPhe³]</td>
<td>21.5 ± 3.2</td>
</tr>
<tr>
<td>6. [Tic²,D-Phe³]</td>
<td>16.5 ± 1.4</td>
</tr>
<tr>
<td><strong>Group C</strong></td>
<td></td>
</tr>
<tr>
<td>7. [Phe²,desGly³]</td>
<td>217.3 ± 16.8</td>
</tr>
<tr>
<td>8. [Tic²,desGly³]</td>
<td>26.0 ± 2.3</td>
</tr>
<tr>
<td><strong>Group D</strong></td>
<td></td>
</tr>
<tr>
<td>9. [Phe²,Gly³]</td>
<td>71.0 ± 6.5</td>
</tr>
<tr>
<td>10. [Tic²,Gly³]</td>
<td>35.2 ± 0.4</td>
</tr>
</tbody>
</table>

* See experimental section for details of the assays.
Table 3.4. Opioid Activity of Dyn A-(1-11) Analogues in the GPI

<table>
<thead>
<tr>
<th>Peptide</th>
<th>IC$_{50}$ in nM$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>[X$^2$Y$^3$,D-Pro$^{10}$]Dyn A-(1-11)NH$_2$</td>
<td>0.27 (0.24 - 0.57)$^a$</td>
</tr>
</tbody>
</table>

**Group A**
1. [Phe$^2$,Phe$^3$] > 10,000
2. [Tic$^2$,Phe$^3$] > 1000
3. [Pip$^2$,Phe$^3$] ~520
4. [N-MePhe$^2$,Phe$^3$] > 1000

**Group B**
5. [Phe$^2$,DPhe$^3$] nd
6. [Tic$^2$,DPhe$^3$] nd

**Group C**
7. [Phe$^2$,desGly$^3$] > 10,000
8. [Tic$^2$,desGly$^3$] > 10,000

**Group D**
9. [Phe$^2$,Gly$^3$] > 1000$^b$
10. [Tic$^2$,Gly$^3$] > 1000$^b$

$^a$ (95 % confidence Interval)

$^b$ No inhibition observed at a dose of 1 μM

nd: not determined
3.8. Conclusions

In this study, we attempted to impart antagonist activity to Dyn A by conformational restriction in the "message" sequence. Recently the tetrapeptide TIPP (Tyr-Tic-Phe-Phe) and the tripeptide TIP (Tyr-Tic-Phe) were reported to be δ antagonists (Schiller et al., 1992a; Schiller, 1993). To achieve the conformational constraint in the "message" sequence, we chose to combine TIPP and TIP sequence with the C-terminal "address" sequence of Dyn A. We also incorporated Pip and NMePhe in position 2 and several modifications in position 3 (Table 3.1) of the TIPP message sequence.

It is clear from our study that position 2 is important in determining opioid receptor and opioid activity. The peptides with a conformationally constrained or hindered amino acid in position 2 showed better affinity for the κ-receptors compared to peptides with Phe in position 2. Additional assays are being conducted to determine the effect of these modifications on receptor selectivity. The preliminary GPI testing to determine opioid activity indicate that most of these peptides possessed weak agonist activity with peptides containing hindered amino acid residues showed slightly higher agonist activity. The Gly³ peptides, however exhibited negligible agonist activity at doses up to 3 μM. These preliminary results suggest that selected peptides may be lead compounds for the development of κ-receptor antagonists.
Acknowledgment

This research was supported by a grant from the National Institute on Drug Abuse (R01 DA05195). We would like to acknowledge the technical assistance of Elizabeth Olenchek for performing the GPI assays and radioligand binding assays. We would also like to thank Jean Pham and Thomas Fallon for the synthesis and purification of control compounds [Phe²,phe³,DPro¹⁰]Dyn A-(1-11) and [Phe²,desGly³,DPro¹⁰]Dyn A-(1-11) respectively. We appreciate Brian Arbogast who performed FAB-MS and LC-MS analysis.
CHAPTER 4

COMPARISON OF COUPLING AGENTS AND RESINS FOR THE EFFICIENT COUPLING OF HINDERED AMINO ACIDS IN SOLID PHASE PEPTIDE SYNTHESIS

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4.1. Abstract

The purity and yield of the desired peptides in solid phase peptide synthesis depends mainly on the peptide’s amino acid sequence and the efficiency of coupling agents and additives. Coupling of hindered amino acid residues using standard reagents is often inefficient and can result in several side products (Ryakhovskii et al., 1991). In order to test the effect of conformational restriction of the N-terminal "message" sequence on opioid activity and opioid receptor selectivity, a series of analogues of the opioid peptide dynorphin A containing hindered amino acids such as Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid) in position 2 were assembled on a polystyrene resin (PAC®) (Chap. 3). Anticipating difficulty in coupling of the hindered amino acids, the usual synthetic strategy was modified for the last two couplings and PyBOP (benzotriazol-1-yl-oxy-tris-pyrrolidinophosphonium hexafluorophosphate) was used in place of DIC (diisopropylcarbodiimide) along with HOBt (1-hydroxybenzotriazole). Still the crude peptides showed considerable amounts of several impurities (characterized by on-line HPLC mass spectrometry) due to incomplete couplings. Therefore we selected a model peptide [Tic2,Phe3,D-Pro10]Dyn A-(1-11)OH, containing the TIPP (Tyr-Tic-Phe-Phe) “message” sequence combined with the Dyn A “address” portion. In order to conduct a comparison of resins we assembled our model peptide on the polystyrene resin (PS) and also the recently introduced polyethyleneglycol-polystyrene resin (PEG-PS) by solid phase synthesis using the Fmoc protocol. The solid support used in solid phase synthesis is an important determinant of high yield and purity of peptides. Our resin study with PS and PEG-PS did not show any significant differences in the components present
in the crude peptide or their relative proportions, but the required coupling times were slightly shorter for the synthesis using the PEG-polystyrene resin.

As a further attempt to minimize the side products and to improve the yield of the desired peptides, we selected the same model peptide [Tic²,Phe³,D-Pro¹⁰]Dyn A-(1-11)OH and the PEG-PS resin, and conducted a comparative study with different activating agents (PyBOP, PyBroP (bromotripyrrolidinophosphonium hexafluoride), HATU (O-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and also coupling additives (HOBt vs HOAt [1-hydroxy-7-azabenzotriazole]). These coupling agents are reported to increase the coupling efficiency in the case of hindered amino acids such as N-methyl amino acids. We found in our study that HOAt is a better additive than HOBt since it reduces coupling times and also reduces undesirable side products. HATU and HOAt together gave the highest purity of the desired peptide along with reducing the coupling times.

Key words: PEG PS resin, coupling agents, coupling additives, hindered amino acids
4.2. Abbreviations

Abbreviations used for amino acids are according to the rules specified by the IU-PAC-IUB Joint Commission of Biochemical Nomenclature in Eur. J. Biochem. 1984, 138, 9-37 and J. Med. Chem. 1992, 35, 3956-3961. All amino acids are in the L configuration unless otherwise specified. Additional abbreviations used are BOP, (benzotriazol-1-yl-oxy-tridimethylaminophosphonium hexafluorophosphate); DCM, dichloromethane; DIC, diisopropylcarbodiimide; DIPEA, N,N'-diisopropylethylamine; DMA, N,N-dimethylacetamide; EDT, ethanedithiol; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenlymethoxycarbonyl; HBTU, (O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate); HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; HOAt, 1-hydroxy-7-azabenzotriazole; HATU, O-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; MBHA, 4-methylbenzhydrylamine; NMePhe, N'-methylphenylalanine; PEG-PS, polystyrene; Pip, pipecolic acid; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; PyBOP, benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; PyBroP, bromotripyrrolidinophosphonium hexafluoride; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.
4.3. Introduction and Rationale

Dyn A is believed to be an endogenous ligand for kappa opioid receptors (Chavkin et al.; 1982). One of the main goals of research performed in our laboratory is the synthesis of dynorphin A (Dyn A) analogues in an attempt to develop κ-receptor selective ligands. In order to test the effect of conformational constraint in the N-terminal “message” sequence (Chavkin et al., 1982; Schwyzer, et al., 1977), we combined a δ selective antagonist, TIPP {((Tyr-Tic-Phe-Phe) where Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid)} (Fig. 4.1) with the Dyn A address and synthesized several [TIPP,D-Pro¹⁰]Dyn A-(1-11) analogues (Chapter 3).

Although solid phase synthesis utilizing the Fmoc synthetic strategy often provides peptides with good yield and high purity, this is not always the case with hindered amino acid residues. Standard coupling reagents are often inefficient for these reactions. Therefore in order to facilitate the coupling of the hindered amino acids, the standard solid phase synthetic strategy was modified slightly for the synthesis of Dyn A analogues (Scheme, Fig. 4.4) and PyBOP was used for the last two couplings (see Experimental Section). However, reversed phase HPLC analysis of all of the crude peptides containing a hindered amino acid residue indicated that they possessed similar impurities. Further LC-MS analysis revealed that the products from each synthesis were identical in nature and proportion (Chapter 3). Most of the side products were due to coupling difficulties with the last two residues. Therefore a model peptide [Tic²,Phe³,D-Pro¹⁰]Dyn A-(1-11) (Fig. 4.1), containing a combination of the TIPP (Tyr-Tic-Phe-Phe) “message” sequence
with the Dyn A address portion was selected for further study to compare the efficiency of coupling agents, additives and resins.

Dynorphin A

H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-D-Pro-Lys-OH

1 2 3 4 5 6 7 8 9 10 11

Message Sequence Address Sequence

Dynorphin A with TIPP Message Sequence

H-Tyr-Tic-Phe-Phe-Leu-Arg-Arg-Ile-Arg-D-Pro-Lys-OH

1 2 3 4 5 6 7 8 9 10 11

Message Sequence Address Sequence

L-Tic

(1,2,3,4-Tetrahydroisoquinoline-3-carboxylic Acid)

Fig. 4.1. Structure of the Model Peptide With the “TIPP” Message Sequence
4.3.1. Comparative Study of Resins

The type of solid support used in solid phase synthesis plays an important role in determining the yield and purity of peptides. The recently introduced PEG-PS [(polyethylene glycol)-polystyrene] supports (Barany, et al., 1992; Mitchell, et al., 1978) uniformly incorporate a derivatized polyethylene glycol spacer between the functional group on the polystyrene (PS) and the handle or attachment to the peptide (Fig 4.3). The PEG-PS resin has the advantage of optimal swelling, which aids in rapid coupling. The PEG-PS exhibits excellent swelling properties compared to PS, especially in DCM; the ratios of swollen/dry volumes are 5.8 for PS and 6.9 for PEG-PS. Therefore in an attempt to minimize the proportions of side products and increase the coupling efficiency, we selected a model peptide [Tic2,Phe3,D-Pro10]Dyn A-(1-11)OH and conducted a comparison of PS (polystyrene) and PEG-PS resins using a slightly modified synthetic strategy for both the resins (Fig. 4.4).

4.3.2. Comparative Study With Different Coupling Agents and Additives

The DIC / HOBt coupling scheme has been a prominent coupling method used in solid phase peptide synthesis, since DIC gives a soluble urea byproduct and HOBt suppresses racemization (Mojsov et al., 1980). But high coupling efficiency may not be achieved with these standard reagents in cases of couplings involving hindered amino acid residues. The phosphonium reagent BOP (1-benzotriazolyloxy-tris(dimethylaminophosphonium hexafluoride) was introduced (Castro, et al., 1975) and was
reported to improve difficult couplings, but its popularity declined because it yields a carcinogenic byproduct, HMPA. This drawback was overcome by the recent introduction of PyBOP (benzotriazol-1-yl-oxy-tris-pyrolidinophosphonium hexafluoride) (Coste, et al., 1990) and PyBroP (bromotripyrolidino-phosphonium hexafluoride) reagents (Coste et al., 1991).

Several “uronium” reagents have been reported to be excellent coupling reagents for peptide bond formation. HBTU (O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and TBTU (O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoroborate) (Knorr et al., 1989) were found to be superior coupling agents in solid phase peptide synthesis. Other recently introduced promising coupling agents are HAPyU (O-7-azabenzotriazol-1-yl-1,1,3,3-bis(tetramethylene)uronium hexafluorophosphate) (Ehrlich et al., 1993), HATU (O-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and the additive HOAt (1-hydroxy-7-azabenzotriazole) (Carpino, 1993). These reagents show excellent coupling efficiency with low levels of racemization (Carpino, 1993).

We selected several coupling agents (PyBOP, PyBroP, HATU) and two coupling additives (HOBt vs HOAt) (Fig. 4.2) which are reported to increase the efficiency of coupling of hindered amino acids (as mentioned earlier) and performed the synthesis of our model peptide ([Tic₂,Phe³,D-Pro¹⁰]Dyn A-(1-11)OH) (Fig. 4.1) using the modified synthetic strategy (Fig. 4.4). The usual DIC / HOBt coupling procedure was used through the coupling of Fmoc-Phe³ and then the last two residues were coupled by four different methods (Fig. 4.4), a) Method I: PyBOP and HOBt, b) Method II: PyBOP and HOAt,
c) Method III: HATU and HOAt, and d) Method IV: PyBroP (see experimental section for the details). The results from the comparison studies for both resins and coupling reagents are discussed below.

![Diagram of coupling agents]

**Fig. 4.2. (a) Coupling Agents**
*Structure of HATU proposed from x-ray experiments (Abdelmoty et al., 1994).*
4.4. Materials and Methods

4.4.1. Materials

PEG-PS resin with the PAC© linker, HATU and PyBroP were purchased from Milipore (Bedford, MA). Other sources of chemical reagents and solvents used for peptide syntheses were the same as described earlier (Chapter 3). The model peptide was assembled on a PS and PEG-PS support using the modified synthetic strategy described previously (Chapter 3). The same model peptide was also synthesized by each of the four methods using different coupling agents and additives (Fig. 4.3) on a Biosearch 9500 automated peptide synthesizer (Novato, CA) as described earlier (Chapter 3). The equipment used for solid phase synthesis, HPLC system for purification and analysis were the same as described earlier in Chapter 3. Analytical HPLC was performed to analyze the crude peptides using a binary solvent system (solvent A, aqueous 0.1% TFA and solvent B AcCN containing 0.1% TFA) with a linear gradient of 0% B to 75% B over...
50 min at a flowrate of 1.5 mL/min, detection at 214 nm. The components present in the crude peptides were characterized by LC-MS ion spray spectrometry performed on a PE-SCIEX, API III, Bimolecular Mass Analyzer, in the Department of Agricultural Chemistry, Oregon State University, Corvallis, OR. A Vydac protein and peptide plus reversed phase C18 column of 1 mm with 5 µm particle size and 300 Å pores size was combined with on-line ion spray mass spectrometry. The ion spray voltage used was 5000 eV and the orifice voltage applied was 80 V. Typically the solvents used were those indicated above for analytical HPLC and analyses were performed using a linear gradient of 10% B to 60%B over 50 min at a flow rate of 40 µL/min. Multiply charged ions were converted to a singly charged ion by automated HyperMass software to obtain the mass of each side product.

4.4.2. Peptide Synthesis for the Comparative Study of Resins

We assembled the model peptide [Tic2,Phe3,D-Pro10]Dyn A-(1-11)OH on a polystyrene resin using the modified synthetic strategy (Chapter 3). In an attempt to minimize the byproducts we also synthesized the same peptide on the PEG-PS resin (0.5 g, 0.19 mmol/g substitution) using the same procedure as that described earlier (Chapter 3). All of the Fmoc-amino acids except Fmoc-Tic and Fmoc-Tyr(tBu) were coupled by the usual DIC / HOBt synthetic strategy. (Chapter 3). Fmoc-Tic and Fmoc-Tyr(tBu) were coupled using PyBOP and HOBt (Chapter 3). Extra DIPEA was added twice during the coupling reactions of both Fmoc-Tic and Fmoc-Tyr(tBu). Double coupling of Fmoc-Tyr(tBu) was
performed in both syntheses. The couplings were monitored by qualitative ninhydrin (Kaiser, et al., 1970) or the chloranil test (Christensen, 1979). The coupling times required for Fmoc-Tic and Fmoc-Tyr(tBu) were 2.5 h and 4 h respectively, in case of PS resin, whereas 2 h and 3.5 h when PEG-PS was used. After the completion of assembly of the peptides, the removal of the N-terminal Fmoc group was performed by the standard deprotection scheme described earlier. The resins were washed with DCM/DMA (1/1, 3x), and MeOH (5x, 15 min) then collected and dried in vacuo.

4.4.3. Peptide Synthesis for the Comparative Study of Coupling Agents

The model peptide [Tic²,Phe³,D-Pro¹°]Dyn A-(1-11)OH, containing TIPP (Tyr-Tic-Phe-Phe) as the “message” sequence combined with the Dyn A address portion was assembled on the PEG-PS resin containing the PAC handle (0.5 g, 0.19 mmol/g substitution). All of the Fmoc-amino acids except Fmoc-Tic and Fmoc-Tyr(tBu) were coupled by the usual DIC / HOBt synthetic strategy, described previously (Chapter 3). Fmoc-Tic and Fmoc-Tyr(tBu) were coupled by four different methods, a) Method I: PyBOP and HOBt, b) Method II: PyBOP and HOAt. c) Method III: HATU and HOAt, d) Method IV PyBroP. (Fig. 4.3).

Briefly, the peptides were assembled in two stages. In the first stage, each Fmoc-protected amino acids (present in a 4-fold excess) was coupled to the growing peptide chain using equimolar amounts of DIC and HOBt in DCM/DMA (1/1 v/v) (final concentrations 0.2 M) for 2 hours. Qualitative ninhydrin color test (Kaiser, et al., 1970) was per-
formed to ensure the completion of the couplings of each residue. Fmoc deprotection was performed in two steps (3 min followed by 7 min) using piperidine/toluene/DMA (30/35/35). This strategy was followed up through the coupling of Fmoc-Phe\textsuperscript{3} and the resin then physically split into four different methods as mentioned below.

4.4.3.1. Method I

A two-fold excess of Fmoc-Tic was mixed with an equimolar amount of HOBt dissolved in DMA (to give a final concentration of 0.2 M) and added to the reaction vessel. Meanwhile PyBOP (1 equivalent) and DIPEA (3 equivalents) were mixed and added to the reaction mixture within one min and the mixture agitated with nitrogen. The pH of the reaction mixture was maintained at approximately 7-8 by addition of extra DIPEA twice. After the qualitative ninhydrin test indicated that the coupling was complete, the deprotection of Fmoc group was performed twice (~15 min each). Fmoc-Tyr(tBu) was then coupled in a similar manner except that a four-fold excess was used (extra DIPEA was added twice). The coupling was checked by the chloranil color test (Christensen, 1979). Fmoc-Tyr(tBu) was double coupled using the same procedure.

4.4.3.2. Method II

This method was identical to Method I except that HOAt was used as an additive in place of HOBt. After qualitative ninhydrin, the two-step deprotection of the Fmoc group
Fmoc-PAC® Resin (For synthesis of peptide acids)

PEG-PS Support
(The handle used was PAC®)

Fig. 4.3. Resins Used in Comparative Study
was repeated manually (~15 min). Then the Fmoc-Tyr(tBu) was double coupled by the procedure discussed above. The coupling was monitored by the chloranil test and also a visual indication given by HOAt (from yellow to colorless) was helpful in determining the endpoint of the reaction (Carpino, 1993).

4.4.3.3. Method III

In this method HATU along with the additive HOAt was used. Fmoc-Tic was mixed with an equimolar amount of HOAt as mentioned above, dissolved in DMA (to give a final concentration of 0.2 M) and added to the reaction vessel. Then HATU (slightly less than 1 equivalent (95 %)) and DIPEA (3 equivalents) were mixed and added to the reaction mixture within one min and the reaction mixture agitated under nitrogen. Care was taken to ensure that HATU reagent was not present in excess and that the free amine was not exposed to the reagent alone (Gausepohl et al., 1991). After monitoring of the coupling with the ninhydrin color test indicated that the coupling was complete, the deprotection of the Fmoc group was performed twice and then Fmoc-Tyr(tBu) was coupled in the same manner, except that a four-fold excess of Fmoc-Tyr(tBu) was used. This coupling was checked by the chloranil color test and visual color change characteristic of HOAt (yellow to colorless). Double coupling of Fmoc-Tyr(tBu) was not required in this synthesis.
Fmoc-Lys(Boc)-(PAC)-PEG-PS resin (R)

1. Deprotection: Piperidine/toluene/DMA (30/35/35, v/v)
2. Coupling: Fmoc-D-Pro+DIC+HOBt (4x excess)
   - Fmoc-D-Pro-Lys(Boc)-R

Repetitive cycles of Fmoc deprotection and DIC/HOBt mediated coupling

Fmoc-Phe-Phe-Leu-Arg(Pmc)-Arg(Pmc)-Ile-Arg(Pmc)-D-Pro-Lys(Boc)-R

Method I Method II Method III Method IV
(PyBOP / HOBt) (PyBOP / HOAt) (HATU / HOAt) PyBroP

Fmoc-(Tic) (2x excess) + Reagent

1. Deprotection using piperidine/toluene/DMA
2. Fmoc-Tyr(tBu) (4x excess) + Reagent
3. Piperidine/toluene/DMA (30/35/35)

Tyr(tBu)-Tic-Phe-Phe-Leu-Arg(Pmc)-Arg(Pmc)-Ile-Arg(Pmc)-D-Pro-Lys(Boc)-R

Reagent K
(TFA + phenol + thioanisole + ethanedithiol)

Tyr-Tic-Phe-Phe-Leu-Arg-Arg-Ile-Arg-D-Pro-Lys-OH

Fig.4.4. Synthetic Strategy for the Model Peptide Using Different Coupling Agents
4.4.3.4. Method IV

The halogenophosphonium reagent PyBroP was used to couple Fmoc-Tic and Fmoc-Tyr in this method. A solution of Fmoc-Tic and Fmoc-Tyr(tBu) (two-fold and four-fold excess, respectively) in DCM (final conc. 0.2 M) were prepared, since this solvent gives better results (Coste, et al., 1994), and added to the reaction vessel. The coupling reagent PyBroP (1 equivalent) was mixed with DIPEA (3 equivalents) in DCM and added to the reaction vessel within 1 min and the contents agitated with nitrogen. The coupling was monitored by the ninhydrin test for Fmoc-Tic and by the chloranil test for Fmoc-Tyr(tBu) as mentioned earlier. Fmoc Tyr(tBu) was double coupled.

After the completion of the peptide assembly, the N-terminal Fmoc group of the peptide obtained from each synthesis was removed by the two step deprotection scheme mentioned earlier and then the resins were washed successively with DCM/DMA (1/1, 3x) and MeOH (5x, 15 min), collected and dried in vacuo.

4.5. Cleavage of the Peptides from the Support

The cleavage reactions were carried out as described earlier (Chapter 3). Briefly, the dried protected resins were treated with 5 mL Reagent K (82.5% TFA, 5% phenol, 5% thioanisole and 2.5% ethanedithiol) under nitrogen at room temperature for about 3 hours. The resin was filtered, washed with TFA, and then the solution was concentrated to about half of the its original volume with nitrogen. The solution was then diluted with 20% * acetic acid (* a higher concentration of acetic acid was used to avoid emulsion for-
formation) (40 mL) and extracted with anhydrous Et$_2$O (3x). The aqueous layers were then lyophilized to give the crude peptides.

The resulting peptides were analyzed by reversed phase analytical HPLC (gradient 0% B-75% B in 50 min at a flow rate of 1.5 mL/min). The components of the crude peptides synthesized by the different methods were characterized by LC-MS, ion spray spectrometry.

4.6. Results

In our earlier study (Chapter 3) we found that the synthesis of a series of Dyn A analogues containing hindered amino acids give rise to deletion sequence side products resulting from incomplete couplings. This study was designed specially to minimize the side products and improve the yield of the desired peptide in these syntheses. We chose a model peptide [Tic$^2$,Phe$^3$,D-Pro$^{10}$]Dyn A-(1-11)OH and conducted a comparative study. In the first part of our study we used PEG-PS resin to assemble the model peptide so as to compare the results with the PS resin (Chap 3). In the second part of this study we synthesized the same model peptide by four different methods using several coupling agents and additives which are reported to be efficient agents for coupling hindered amino acids. We characterized the products of the syntheses by analytical HPLC and LC/MS.
4.6.1. Identification of the Side Products

The combination of conventional HPLC technique with on-line ion spray mass spectrometry (LC/MS) was found to be useful to characterize the side products in our syntheses (Table 4.1). The analytical reversed phase HPLC for the peptides synthesized using method I to IV indicated formation of similar side products but with considerable differences in the proportions (Fig. 4.5, Table 4.2). In general there were three major side products observed from these syntheses, along with the major desired peptide (1554.9 u). Total ion currents (TIC) of all masses between 330 to 1600 were collected and are shown in the chromatograms in Fig. 4.6. For each peak, we obtained multiply charged ions and from which molecular weight was determined by hypermass calculation (Table 4.1). In addition to the desired peptide (1554.9 u) two side products with 1391.7 u and 1231.9 u were found. A third side product (1433.7 u) was also observed in the LC-MS (Table 4.1).

The major product in each synthesis was the desired peptide (1554.9 u). Two other products were identified as products of incomplete couplings, one due to incomplete coupling of Tyr (des-Tyr, 1391.7 u) and the other from incomplete couplings of both Tyr and Tic (des-Tyr,des-Tic; 1231.9 u). A third side product (1433.7 u) was also observed. The quantity of this last product isolated following purification was very low (<1 mg.); therefore we did not investigate the structure of this product further. The comparison of products of synthesis from each method is are summarized in Table 4.2 and Fig. 4.7.
Table 4.1. Identity of Products Using Hypermass Calculations From LC/MS
(All the methods used in synthesis of the model peptide gave similar products).

<table>
<thead>
<tr>
<th>Compound Identity</th>
<th>Expected MW</th>
<th>Found MW</th>
<th>Charges</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desired peptide</td>
<td>1554.91</td>
<td>1554.9</td>
<td>+2, +3, +4</td>
</tr>
<tr>
<td>Des-Tyr</td>
<td>1391.71</td>
<td>1391.6</td>
<td>+2, +3, +4</td>
</tr>
<tr>
<td>Des-Tyr, des-Tic</td>
<td>1231.9</td>
<td>1232.1</td>
<td>+2, +3</td>
</tr>
<tr>
<td>Other (Identity uncertain)</td>
<td>1433.7</td>
<td>1433.1</td>
<td>+2, +3</td>
</tr>
</tbody>
</table>

Table 4.2. Comparison of Products From Different Synthetic Methods.
(Peptides were assembled on PEG-PS resin unless otherwise specified)

<table>
<thead>
<tr>
<th></th>
<th>(PyBOP + HOBt)*</th>
<th>(PyBOP + HOBt)</th>
<th>(PyBOP + HOAt)</th>
<th>(HATU + (PyBroP) HOAt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Desired peptide</td>
<td>40.3</td>
<td>40.8</td>
<td>38.9</td>
<td>83.2</td>
</tr>
<tr>
<td>% Des-Tyr, des-Tic</td>
<td>10.3</td>
<td>8.5</td>
<td>&lt; 0.5%</td>
<td>Absent</td>
</tr>
<tr>
<td>% Des-Tyr</td>
<td>32.6</td>
<td>27.6</td>
<td>21.5</td>
<td>6.5</td>
</tr>
<tr>
<td>% Other (Unidentified) (Actual wt&lt; 1%)</td>
<td>18.3</td>
<td>22.9</td>
<td>29.5</td>
<td>10.2</td>
</tr>
</tbody>
</table>

* Peptide assembled on PS (polystyrene) resin

♦ Isolation of this side product yielded very small amounts (< 1 mg)
(Proportion of products calculated from peak areas obtained by HPLC at 214 nm)
Table 4.3. Comparison of Coupling Times For Different Synthetic Methods

<table>
<thead>
<tr>
<th></th>
<th>PyBOP +</th>
<th>PyBOP +</th>
<th>(PyBOP +</th>
<th>(HATU +</th>
<th>PyBroP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HOBt*</td>
<td>HOBt</td>
<td>HOAt</td>
<td>HOAt</td>
<td></td>
</tr>
<tr>
<td>Coupling time for</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fmoc-Tic</td>
<td>2.5 h</td>
<td>2 h</td>
<td>1 h</td>
<td>20-25 min.</td>
<td>2.5 h</td>
</tr>
<tr>
<td>Coupling time for</td>
<td>Double</td>
<td>Double</td>
<td>Double</td>
<td>Single</td>
<td>Double</td>
</tr>
<tr>
<td>Fmoc-Tyr(tBu)</td>
<td>(3+1.5) h</td>
<td>(3+1) h</td>
<td>(3+1) h</td>
<td>(2 h)</td>
<td>(3+1) h</td>
</tr>
</tbody>
</table>

* Double coupling times indicated in parenthesis.
* Peptide assembled on PS (polystyrene) resin
Method I: PyBOP + HOBt

Method II: PyBOP + HOAt

Method III: HATU + HOAt

Method IV: PyBroP

Fig. 4.5. Analytical HPLC Profiles for Methods I-IV
Method I: PyBOP + HOBt
Method II: PyBOP + HOAt
Method III: HATU + HOAt
Method IV: PyBroP

Fig. 4.6. TIC Chromatograms for Crude Peptides from Methods I-IV
Fig. 4.7. Comparison of proportions of products
(Using Peak Areas obtained by analytical HPLC at 214 nm)
* Peptide assembled on PS resin
Fig. 4.8. Comparison of Coupling Times
* Peptide assembled on PS resin (PEG-PS used in other cases)
4.6.2. Discussion

The synthesis of our model peptide [Tic₂,Phe³,D-Pro¹⁰]Dyn A-(1-11)OH by all four methods (I-IV) using PS resin and PEG-PS resin resulted in products which contained the desired peptide as the major product (1554 u), and the two main side products des-Tyr (1391 u) and (des-Tic, des-Tyr) (1232 u). When HOAt (method II) was used, the side product (des-Tic, des-Tyr) (1232 u) was almost eliminated and the proportion of des-Tyr (1391 u) was found to be reduced from 27 % to 21 %. compared to HOBt (method I). When the HATU coupling agent was used with HOAt as an additive(method III) the side product (des-Tic, des-Tyr) (1232 u) was not found and the side product des-Tyr (1391 u) was reduced significantly from 27 % to 6.5 %, yielding the desired peptide in highest purity. PyBroP method (IV) gave results very similar to those obtained with PyBOP with no significant differences in the proportion of side products.

The coupling times were found to be shorter for the PEG-PS resin compared to PS resin. The comparison of coupling times for the Fmoc-Tic and the Fmoc-Tyr(tBu) in all the methods are shown in Table 4.3. The coupling times required to give negative ninhydrin or chloranil tests were different for each method. In second method, when HOAt was used as an additive in place of HOBt with PyBOP, the Fmoc-Tic coupling time was decreased (1 h), but there was no effect on the Fmoc-Tyr(tBu) coupling. When HATU + HOAt were used together the coupling times for the Fmoc-Tic decreased significantly (20-25 min) and the Fmoc-Tyr(tBu) coupling was achieved in one coupling (2h) (Table 4.3, Fig 4.6). The required coupling times with PyBroP were slightly longer than that required with PyBOP.
4.7. Conclusions

Incorporation of hindered amino acid residues into peptides can be difficult mainly due to steric hindrance or conformational factors, and deletion sequences are often obtained due to incomplete couplings. Since high purity and yield are very important factors in solid phase synthesis, we performed a comparative study of several coupling agents and additives. We also compared the solid phase peptide synthesis on a PS and PEG-PS resin (both having the PAC® handle). Our model for this study [Tic²,Phe³,D-Pro¹⁰]Dyn A-(1-11)OH, was synthesized by four different methods to compare the coupling efficiency of different agents. Method II (PyBOP + HOAt) was observed to be superior to Method I (PyBOP + HOBt), since the proportions of side products decreased and the required coupling time of Fmoc-Tic was reduced when HOAt was used as an additive. This supports the results found by Carpino (Carpino, 1993). HATU along with HOAt (Method III) gave the desired peptide with the highest purity and the shortest coupling times. These findings also are in accordance with recent reports for methylated amino acids in shorter peptides (Carpino, et al., 1994; Angell, et al., 1994). In our study with PyBroP we did not obtain high yields of the desired peptide. This is in accordance with the results of another study using PyBroP where it did not give good results in solid phase synthesis (Coste, et al., 1994).

This study indicated that the use of HATU as a coupling agent and HOAt as a coupling additive for the synthesis of peptides containing hindered amino acids can increase the yields of the desired peptide by minimizing the side products and reducing the reaction times.
Acknowledgements

This research was supported by a grant from the National Institute of Drug Abuse (R01 DA 05195). We would like to thank Dr. Brian Arbogast for performing LC/MS analysis. We would also like to thank Steven Kates for the gift of HATU and HOAt samples.
CHAPTER 5

CONCLUSIONS

In the past few years, the design and development of opioid peptide analogues has been directed more towards enhancing receptor selectivity. Introduction of a conformational constraint in the backbone of endogenous peptides is a popular strategy and one which has high potential to improve receptor selectivity is used to design the peptide analogues. Dynorphin A (Dyn A) is known to interact preferentially with κ-receptors, but it also shows considerable affinity for μ and δ receptors. The N-terminal tetrapeptide “message” sequence of Dyn A, proposed by Glodstein (Chavkin and Goldstein, 1981) is thought to be important in determining opioid activity and the unique C-terminal “address” sequence imparts high affinity for kappa opioid receptors.

In order to explore the concept of the “message-address sequence” and its importance in opioid activity and receptor selectivity, we synthesized a series of dynorphin A analogues with modifications in the “message” sequence. A tetrapeptide TIPP (Tyr-Tic-Phe-Phe) and tripeptide TIP (Tyr-Tic-Phe-Phe), which are composed entirely of aromatic residues showed selective δ-receptor antagonist activity. The TIPP sequence was chosen as the model for the “message” sequence and we combined it with the address sequence of Dyn A in an attempt to develop κ-receptor selective antagonists. We also performed several modifications in position 2 and 3 of the TIPP sequence. We restricted the conformation by incorporating rigid and hindered amino acid residues like Tic, Pip and NMePhe in position 2. A change in stereochemistry can result in a change of activity.
Therefore we synthesized analogues containing D-amino acids (D-Phe) in position 3. Analogues with a D-amino acid were also synthesized and are being investigated (see Appendix). To find out the importance of the aromatic residue in position 4 of TIPP, we also truncated this sequence by deleting residue 3 (desGly³). We found in our study that peptides with a conformationally constrained or hindered residue in position 2 possessed better κ-receptor affinity compared to the corresponding control compounds containing Phe at position 2. The μ and δ binding assays are in progress. The preliminary GPI testing indicated that these peptides possess weak to negligible (IC₅₀ > 500 nM) agonist activity.

Some selected peptides have been examined for antagonist activity and found to show weak antagonist activity in preliminary experiments. Further experiments to confirm the pharmacological data are in progress.

The coupling difficulty of hindered amino acids gave several side products due to incomplete couplings in the synthesis of Dyn A analogues. In order to increase the yield of the pure desired material, we conducted a comparative study of resins, coupling additives and coupling agents. We selected a model peptide [Tic²,Phe³,D-Pro¹⁰]Dyn A-(1-11)OH and synthesized it first by using PS resin and PEG-PS resin. We found the coupling times slightly shorter when PEG-PS was used but the side products due to incomplete couplings were seen in both the syntheses. Therefore we further did a comparative study with different additives (HOBt vs HOAt) and different coupling agents (PyBOP, PyBroP and HATU). In our study, we found strong evidence that HOAt is a more efficient additive than HOBt, supporting the earlier work done by Carpino (1993). We also found that the use of the HATU along with HOAt minimizes the side products due to in-
complete couplings of hindered amino acids. The reagent PyBroP gave results similar to that of PyBOP. We can therefore conclude that the use of the coupling agent HATU and HOAt (an additive) is desirable for the couplings of hindered amino acids, minimizing side reactions and reducing reaction times.

In conclusion our study supported the hypothesis that the "message" sequence of Dyn A seems to be important for opioid activity. Some of these peptides may be lead compounds with significant antagonist activity. Also the HATU and HOAt coupling scheme appears to be beneficial to minimize the side reactions during the couplings of hindered amino acids.


Lyttle, M. H., and Hudson, D., Peptides: Chemistry, and Biology, J. A. Smith


APPENDIX

Stereochemical Substitution

Incorporation of D-amino acids in place of L-amino acid residues is a common strategy to determine the importance and necessity of specific residues for peptide-receptor interaction (Hruby, 1982). It also helps preventing enzymatic breakdown of peptides, thereby increasing their stability. Therefore we synthesized several dynorphin A analogues with the modifications in the message sequence (Chapter 3). This included the analogues with DPhe in position 3 (Phe²,DPhe³ and Tic²,DPhe³). We further expanded the concept of D-amino acid and study to residue 2 and synthesized two peptides with DPhe in position 2. Depending upon the results of binding affinity and opioid activity for these compounds, we propose to extend the similar series of analogues as described in chapter 3, with D-Phe in position 3. The materials, synthetic method, including purification method used for these peptides is similar to that described in Chapter 3. These peptides were characterized by FAB-MS, analytical HPLC and amino acid analysis (shown in the Table on next pages). The results of pharmacological testing for these peptides are awaited.
Table 6.1. Characterization of Dyn A Analogues Containing D-amino Acids in Position 2

\[\text{[DPhe}^2,\text{Phe}^3,\text{D-Pro}^{10}] \text{Dyn A-(1-11) and [DPhe}^2,\text{D-Phe}^3,\text{D-Pro}^{10}] \text{Dyn A-(1-11)}\]

General compound structure:

\[\text{[(Tyr}^1, X^2, Y^3, \text{Phe}^4), \text{D-Pro}^{10}] \text{Dyn A-(1-11)}\]

\[\text{Tyr}^1 \cdot X^2 \cdot Y^3 \cdot \text{Phe}^4 \cdot \text{Leu}^5 \cdot \text{Arg}^6 \cdot \text{Arg}^7 \cdot \text{Ile}^8 \cdot \text{Arg}^9 \cdot \text{D-Pro}^{10} \cdot \text{Lys}^{11} \cdot \text{OH}\]

\(X = \text{DPhe}\) and \(Y = \text{Phe} / \text{DPhe}\)

<table>
<thead>
<tr>
<th>[DPhe(^2),Phe(^3),D-Pro(^{10})] Dyn A-(1-11)</th>
<th>Gradient(^a) HPLC Rt</th>
<th>FAB-MS M+1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. DPhe(^2),Phe(^3)</td>
<td>25.4</td>
<td>1543</td>
</tr>
<tr>
<td>2. DPhe(^2),D-Phe(^3)</td>
<td>26.1</td>
<td>1543</td>
</tr>
</tbody>
</table>

\(a\)Conditions: Vydac C\(_{18}\) reverse phase column, 0% B to 75% B (0.1% TFA in AcCN) over 50 min at 1.5 mL/min.

Table 6.2. Amino Acid Analysis of Dyn A (1-11) Analogues

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Tyr(1)</th>
<th>Phe(3)</th>
<th>Leu(1)</th>
<th>Arg(3)</th>
<th>Ile(1)</th>
<th>DPro(1)</th>
<th>Lys(1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. D-Phe(^2)-Phe(^3)</td>
<td>1.00</td>
<td>3.04</td>
<td>1.03</td>
<td>2.97</td>
<td>0.92</td>
<td>0.98</td>
<td>1.01</td>
</tr>
<tr>
<td>2. D-Phe(^2)-D-Phe(^3)</td>
<td>1.00</td>
<td>3.04</td>
<td>1.03</td>
<td>2.96</td>
<td>0.92</td>
<td>1.04</td>
<td>1.02</td>
</tr>
</tbody>
</table>
Table 6.3. Opioid Receptor Binding Affinities for Dyn A-(1-11) Analogues Containing D-amino Acids in Position 2

\[ [X^2, Y^3, D-Pro^{10}] \text{Dyn A (1-11) Analogues } K_i (\text{nM}).^a \]

<table>
<thead>
<tr>
<th>[X^2, Y^3, D-Pro^{10}] Dyn A (1-11) Analogue</th>
<th>[^3\text{H}]\text{Bremazocine}</th>
<th>K_i (\text{nM})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dyn A- (1-11)</td>
<td></td>
<td>0.59 ± 0.22</td>
</tr>
<tr>
<td>1. D-Phe^2-Phe^3</td>
<td></td>
<td>13.02</td>
</tr>
<tr>
<td>2. D-Phe^2-D-Phe^3</td>
<td></td>
<td>10.9</td>
</tr>
</tbody>
</table>

^a See experimental section (Chapter 3) for details of the assays.
Table 6.4. Opioid Receptor Binding Data (Updated) for [X^2,Y^3,D-Pro 10]Dyn A-(1-11) Analogues \( K_i \) (nM).^

<table>
<thead>
<tr>
<th>Analogue</th>
<th>( K_i ) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \kappa )</td>
</tr>
<tr>
<td>Dyn A-(1-13)NH₂</td>
<td>0.53 ± 0.35</td>
</tr>
<tr>
<td><strong>Group A</strong></td>
<td></td>
</tr>
<tr>
<td>1. [Phe²,Phe³]</td>
<td>100.1 ± 24.0</td>
</tr>
<tr>
<td>2. [Tic²,Phe³]</td>
<td>20.8 ± 3.9</td>
</tr>
<tr>
<td>3. [Pip²,Phe³]</td>
<td>16.5 ± 0.8</td>
</tr>
<tr>
<td>4. [N-MePhe²,Phe³]</td>
<td>15.9 ± 3.7</td>
</tr>
<tr>
<td><strong>Group B</strong></td>
<td></td>
</tr>
<tr>
<td>5. [Phe²,D-Phe³]</td>
<td>21.5 ± 3.2</td>
</tr>
<tr>
<td>6. [Tic²,D-Phe³]</td>
<td>16.5 ± 1.4</td>
</tr>
<tr>
<td><strong>Group C</strong></td>
<td></td>
</tr>
<tr>
<td>7. [Phe²,desGly³]</td>
<td>217.3 ± 16.8</td>
</tr>
<tr>
<td>8. [Tic²,desGly³]</td>
<td>26.0 ± 2.3</td>
</tr>
<tr>
<td><strong>Group D</strong></td>
<td></td>
</tr>
<tr>
<td>9. [Phe²,Gly³]</td>
<td>71.0 ± 6.5</td>
</tr>
<tr>
<td>10. [Tic²,Gly³]</td>
<td>42.6 ± 7.8</td>
</tr>
</tbody>
</table>

* See experimental section for details of the assays.
* (Assays for \( n = 1 \))