AN ABSTRACT OF THE THESIS OF

Seksiri Arttamangkul for the degree of Doctor of Philosophy in Pharmacy presented on February 9, 1995. Title: Synthesis and Opioid Activity of Conformationally Constrained Dynorphin A Analogues.

Redacted for Privacy

Abstract approved: _____

Jane V. Aldrich

Cyclic analogues of dynorphin A-(1-13) amide (Dyn A-(1-13)NH_2) were synthesized in order to investigate possible biological conformations of this peptide. The design was based upon Schwyzer's hypothesis that Dyn A-(1-13) can adopt an α-helix when it binds to κ opioid receptors. The cyclo[D-Asp^i,Dap^{i+3}]Dyn A-(1-13)NH_2 (Dap = α,β-diaminopropionic acid) analogues were designed based on molecular modeling which suggested that this constraint may be compatible with an α-helix. The cyclic analogues prepared in this study were cyclo[D-Asp^2,Dap^5]-, cyclo[D-Asp^3,Dap^6]-, cyclo[D-Asp^5,Dap^8]- and cyclo[D-Asp^6,Dap^9]Dyn A-(1-13)NH_2. Analogues containing Dab (α,γ-diaminobutyric acid) and Orn in position $i+3$ were also synthesized to examine the effects of a larger ring size. These cyclic analogues exhibited marked differences in opioid binding affinity and opioid activity. The differences were related to the regions in Dyn A where the constraint was incorporated. Of these cyclic constraints, cyclic [2,5] analogues exhibited the highest κ receptor affinity and the highest opioid activity in the guinea pig ileum (GPI), while the cyclic [3,6] analogues exhibited the weakest κ receptor affinity as well as very weak opioid activity in the GPI. The cyclic [5,8] and cyclic [6,9] Dyn A analogues

exhibited moderate to high κ receptor affinity. The opioid activity in the GPI of the cyclic [6,9] analogues was also high, but the opioid activity in the GPI of the cyclic [5,8] analogues was very weak. Changes in ring size did not cause large changes in either opioid binding affinity or opioid activity. Conformational analysis using circular dichroism in 80% trifluoroethanol indicated that cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2 could adopt some helical structure. The helical content of cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2 was slightly higher than that of Dyn A-(1-13)NH_2.

Syntheses of the cyclic Dyn A analogues in this study proved to be difficult and cyclizations generally required several days to get nearly complete reactions. Activating reagents (HBTU, HATU, HAPyU, BOP, PyBOP and PyAOP) were therefore evaluated for their efficiency and applicability for lactam formation in the model Dyn A analogues. The results from various cyclization conditions suggested that these activating reagents could affect both the rate of the cyclization and the purity and yield of the desired cyclic peptide. Cyclizations using HBTU, HATU or HAPyU consistently gave alkylguanidinium byproducts and low yields of the desired cyclic peptides. In contrast, the cyclic peptide products could be obtained in higher yields when cyclizations were performed using BOP, PyBOP or PyAOP, even though these cyclizations were slow. For the cyclization study, HPLC with on-line mass spectrometry and tandem mass spectrometry were used to identify peptide products. Both mass spectrometric methods proved to be useful and convenient techniques for peptide analysis of crude reaction mixtures without purification.

Synthesis and Opioid Activity of Conformationally Constrained Dynorphin A Analogues

by

Seksiri Arttamangkul

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed February 9, 1995

Commencement June 1995

Doctor of Philosophy thesis of Seksiri Arttamangkul presented on February 9, 1995

APPROVED:

Redacted for Privacy

Major Professor, representing College of Pharmacy

Redacted for Privacy

Dean of College of Pharmacy

Redacted for Privacy

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Redacted for Privacy

Seksiri Arttamangkul, Author

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Dr. Jane V. Aldrich for her inspirational guidance, encouragement, assistance and wisdom in peptide chemistry and opioid area. I also want to thank her for her advice and time which we worked together, and for allowing the financial possibility for me to complete the education.

Thanks are also due to Dr. Thomas F. Murray and Dr. Gary E. DeLander for their contributions and guidance in pharmacological works. Thanks are extended to those pharmacologists who performed all the binding and biological assays: Dr. Valery Caldwell, Jane Roth, Barbara Hittinger-smith, Sabrina Zhoa, Martin Knittel and Elizabeth Olenchek.

There are several people who deserve a great deal of credit. Dr. Brian Arbogast, Donald Griffin and Dr. Ole Janssen performed excellent jobs for mass spectrometry experiments and also taught me to use computer software for these analyses. Barbara Robbins performed all amino acid analysis and peptide sequencing.

I would like to express my appreciation to Dr. W. Curtis Johnson for his generous advice in circular dichroism and allowing me to work in his laboratory. Jeannine Lawrence should also be recognized for her technical assistance with the CD instrument.

Special thanks are due to Dr. Sandra B. Story and Dean Maeda for their helps in the laboratory during the past years. The faculty and staff of college of Pharmacy have been wonderful colleagues and friends.

Thanks are also to my committee members: Drs. Aldrich, Block, Gillis, McFadden and Murray. Their time and assistance are gratefully acknowledged.

Lastly, I would like to acknowledge my parent, Swasdi and Somsri Arttamangkul, for their endless support which was very important for the completion of my education. A great deal of moral support has also come from my husband, Dr. Chun-tien Yeh, who encouraged the effort and shared in all pain and fun during these years.

CONTRIBUTION OF AUTHORS

Dr. Jane V. Aldrich dedicated her efforts in advising the research and editing all of the manuscripts. Dr. Thomas F. Murray and Dr. Gary E. DeLander involved in parts of pharmacological evaluations and editing manuscripts which appear in chapters 6 and 7. Dr. Douglas Barofsky and Dr. Brian Arbogast assisted in mass spectrometry experiments. Dr. David Grandy provided the cloned opioid receptors for binding assays. Ms. Jane Roth involved in the radioligand binding assays using the cloned opioid receptors in Dr. Murray's laboratory. Ms. Leena Leelasvatanakij helped to performed the peptide resin [Asp^2,Lys^6]Dyn A-(1-13) that could be used for cyclization study.

TABLE OF CONTENTS

TABLE OF CONTENTS, CONTINUED

TABLE OF CONTENTS, CONTINUED

TABLE OF CONTENTS, CONTINUED

LIST OF FIGURES

LIST OF FIGURES, CONTINUED

LIST OF FIGURES, CONTINUED

LIST OF TABLES

LIST OF TABLES, CONTINUED

ABBREVIATIONS:

Boc	*tert*-butyloxycarbonyl
BOP	benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate
CD	circular dichroism
CHO	Chinese hamster ovary
ClZ	2-chlorobenzyloxy-carbonyl
Dab	α,γ-diaminobutyric acid
DAMGO	[D-Ala^2,MePhe^4,Gly-ol]enkephalin
Dap	α,β-diaminopropionic acid
DCC	*N,N'*-dicyclohexylcarbodiimide
DCM	dichloromethane
DIC	*N,N'*-diisopropylcarbodiimide
DIEA	*N,N*-diisopropylethylamine
DMA	*N,N*-dimethylacetamide
DMF	*N,N*-dimethyl-formamide
DPDPE	cyclo[D-Pen^2,D-Pen^5]enkephalin
Dpg	dipyrrolidinylguanidinium
DPPA	diphenylphosphorylazide
Dyn A	dynorphin A
FAB-MS	fast atom bombardment mass spectrometry
Fmoc	(9-fluorenylmethoxy)carbonyl
Fmoc-OSu	9-fluorenylmethyl succinimidyl carbonate

ABBREVIATIONS, CONTINUED:

GPB	guinea pig brain
HAPyU	*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(tetramethylene)uronium hexafluorophosphate
HATU	*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HBTU	*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
MBHA	4-methylbenzhydrylamine
NMR	nuclear magnetic resonance
PAL® resin	Peptide Amide Linker or 5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid resin
Pmc	2,2,5,7,8-pentamethylchroman-6-sulfonyl
PyAOP	7-azabenzotriazol-1-yl-oxy-trispyrrolidinophosphonium hexafluorophosphate
PyBOP	benzotriazol-1-yl-oxy-trispyrrolidinophosphonium hexafluorophosphate
TBTU	*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TFA	trifluoroacetic acid
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
THF	tetrahydrofuran

ABBREVIATIONS, CONTINUED:

Tmg	tetramethylguanidinium
Tos	4-toluenesulfonyl
Z	benzyloxycarbonyl
GPI	guinea pig ileum

SYNTHESIS AND OPIOID ACTIVITY OF CONFORMATIONALLY CONSTRAINED DYNORPHIN A ANALOGUES

CHAPTER 1
INTRODUCTION

1.1 Background and motivation

Opium alkaloids are one of the oldest medicines known [Brownstein, 1993]. They have been primarily used as analgesics, but they also produce various pharmacological effects including addiction, tolerance and physical dependence. The discovery of opioid receptors and their endogenous ligands has inspired many scientists to study the relationships between these receptors and their ligands in efforts to resolve the complexities of the physiological and pharmacological roles of opioid receptors. Difficulties in discerning the biological effects produced by different opioid receptor types are related to the multiplicity of opioid receptors and their endogenous ligands, and to the low selectivities of the endogenous opioid ligands for individual receptor types [Simon and Hiller, 1994]. The development of highly selective ligands for each receptor type is therefore a major goal in opioid research.

During the last decade it has been suggested that selective agonists for κ opioid receptors may represent potential analgesics without significant clinical side-effects [Millan, 1990; Rees, 1992]. Understanding the topological requirements of ligands for κ receptors will be valuable in the design of κ-selective ligands. Since dynorphin A (Dyn A) has been postulated to be an endogenous ligand for κ receptors, information about the conformation Dyn A may adopt at κ receptors will be useful for determination of the topological requirements of κ receptors.

1.2 Objectives

The objective of this dissertation was to use the hypothesis that Dyn A adopts a helical structure at κ opioid receptors [Schwyzer, 1986a] to design conformationally constrained Dyn A analogues. A conformational constraint via side-chain to side-chain cyclization was incorporated into Dyn A-(1-13)NH_2 to reduce the peptide's flexibility. The constraint was designed based upon molecular modeling which suggested that a four-atom bridge between the side-chains of D-Asp^i and Dap^{i+3} (Dap = α,β-diaminopropionic acid) could be compatible with a helical structure (see more detail below).

Four series of cyclic Dyn A-(1-13)NH_2 analogues (Figure 1.1) were synthesized using a solid phase synthetic strategy. All of the cyclic Dyn A-(1-13)NH_2 analogues and their corresponding linear peptides were evaluated for opioid binding affinity and opioid activity (see chapters 6 and 7). The results from these assays could indicate compatibility of the constraints and modifications of these Dyn A analogues with opioid receptors. The opioid binding affinities of these peptides for κ, μ and δ receptors were determined using either homogenized brain membranes or cloned receptors expressed in COS-7 or CHO cells by Dr. Thomas Murray and his coworkers. The opioid activities of these analogues were evaluated using guinea pig ileum (GPI) assays by Dr. Gary DeLander and his coworkers. Preliminary conformation analysis of one cyclic peptide was performed using circular dichroism.

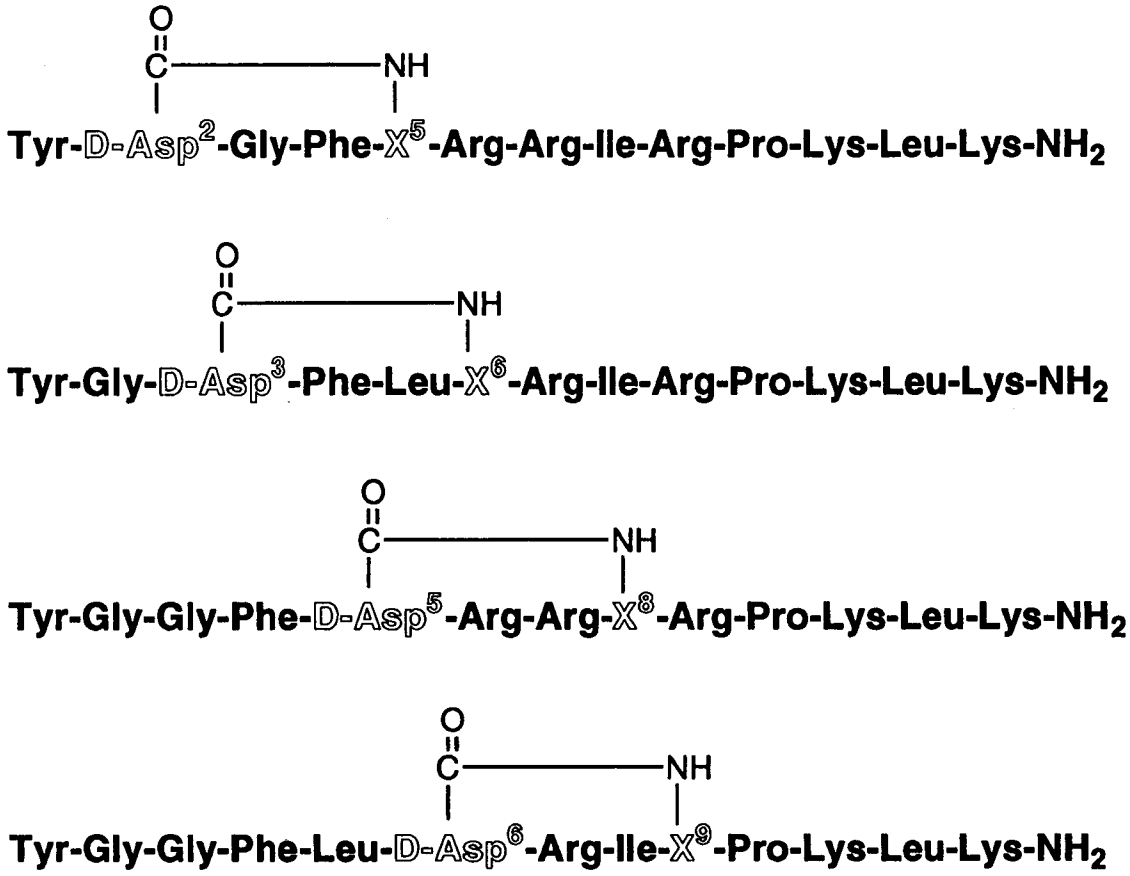


Figure 1.1 Four series of cyclic Dyn A-(1-13)NH_2 analogues

In the synthesis of a cyclic peptide, the cyclization reaction is commonly the yield-limiting step. Optimization of the cyclization procedure is therefore required in order to obtain the desired cyclic peptide in high purity and yield. In this study the phosphonium and the uronium activating (coupling) reagents were evaluated to determine the efficiency and applicability of these reagents for the cyclization (see chapter 4). The peptide products obtained from the cyclization study were identified using HPLC with on-line ion spray mass spectrometry and ion spray tandem mass spectrometry (see chapter 5). These two new methods were expected to provide advantages for analysis of synthetic peptides over the conventional analyses, i.e. reverse phase HPLC, off line FAB-MS, amino acid analysis and Edman sequencing.

1.3 Rationale

There have been several possible conformations reported for Dyn A [Renugopalakrishnan et al., 1990], but a promising conformation proposed by Schwyzer [1986a] is that Dyn A adopts an α-helix at κ receptors. In Schwyzer's hypothesis, Dyn A-(1-13) possesses a helical structure from Tyr^1 through Arg^9 with an unordered structure from Pro^{10} through Lys^{13} [Schwyzer, 1986a]. Schwyzer also speculated that there is membrane compartmentalization for opioid receptors and suggested that κ opioid receptors are buried in a lipid membrane bilayer [Schwyzer, 1986b]. The negative charges (10 or 11 residues) existing on the extracellular domains of the cloned κ receptors may promote the accumulation of Dyn A in the immediate vicinity of the binding sites. Then amino acid residues 1 - 9 of Dyn A-(1-13) could induce a helical structure as a consequence of the peptide's significant

amphiphilic moment, $\overrightarrow{A}$, and insert into the hydrophobic membrane compartment [Schwyzer, 1992; 1995].

Experimental evidence for an α-helix of Dyn A has also been reported. Taylor [1990] reported evidence for 20% helical content in Dyn A by simulating the CD spectrum with a mixture of α-helical and a random coil peptide in a molar ratio of 20 to 80, respectively. Using 2D-NMR and molecular modeling, Epand and his coworkers [1992] found a helical structure for residues Tyr^1 to Leu^5 and a folded structure for residues Ile^8 to Pro^{10}. Recently, Kallick [1993] has evaluated the structure of Dyn A in dodecylphosphocholine micelles using 2D-NMR (500 MHz) and reported that this peptide adopts a helix from Gly^3 to Arg^9.

1.4 Designs for stabilizing an α-helix in Dyn A

1.4.1 Background

Attempts to generate short α-helical peptides in solution have had limited sucess so far due to the nature of helical structure initiation and stabilization; hence non-native amino acids, and side-chain or backbone bridges are often used as elements for stabilizing helices in the peptide sequences. These include: (1) incorporation of C^{α}-alkylated amino acid residues into an oligopeptide chain [Toniolo et al., 1993]; (2) incorporation of trifunctional amino acids (e.g. His or Cys at positions i and $i+4$) into the peptide chain, allowing helix stabilization via complexation of amino acid side-chains with a transition metal [Ghadiri and Choi, 1992]; (3) helix stabilization by charged groups on an amino acid side-chain (e.g.

Asp, Glu or Lys) interacting with the α helix dipole [Shoemaker et al., 1987]; (4) helix stabilization by salt bridges between Glu^- and Lys^+ at positions *i* and *i+4* [Marqusee and Baldwin, 1987]; and (5) conformational constaints using cyclization between side-chains of certain residues in the peptide chain [Felix et al., 1988; Ösapay and Taylor, 1992; Miranda et al., 1994].

Additionally, several rigid building blocks have been utilized to geometrically fix the first amino acid in the proper orientation for helix initiation. These constraints include: 1,6-disubstituted indanes [Nolan et al.,1992], diacyl-azabicyclo [2,2,2] octanone derivatives [Müller et al., 1992] and the triacid derivatives of Kemp [Kemp and Curran, 1988]. Recently, a conformationally constrained bicyclic hexapeptide containing two overlapping *i* (Lys) to *i+4* (Asp) side-chain lactam bridges has been synthesized and found to be useful as an α-helical peptidomimetic scaffold or α-helix initiator [Bracken et al., 1994].

1.4.2 α-Helix design in this study

A novel design for stabilizing an α-helix in this study was proposed in our laboratory using the molecular modeling program AMBER [Weiner and Kollman, 1981; Weiner et al., 1984; Weiner et al., 1986]. Using polyalanine as a model peptide, this molecular modeling suggested that a lactam bridge between the side-chains of residues *i* (D-configuration) and *i+3* (L-configuration) containing a four-atom bridge may be compatible with an α-helix. To establish the desired cyclic structure, D-Asp and Dap were selected for residues *i* and *i+3*, respectively (Figure 1.2). The idealized C^α - C^α distance between residues i and i+3 in an α-

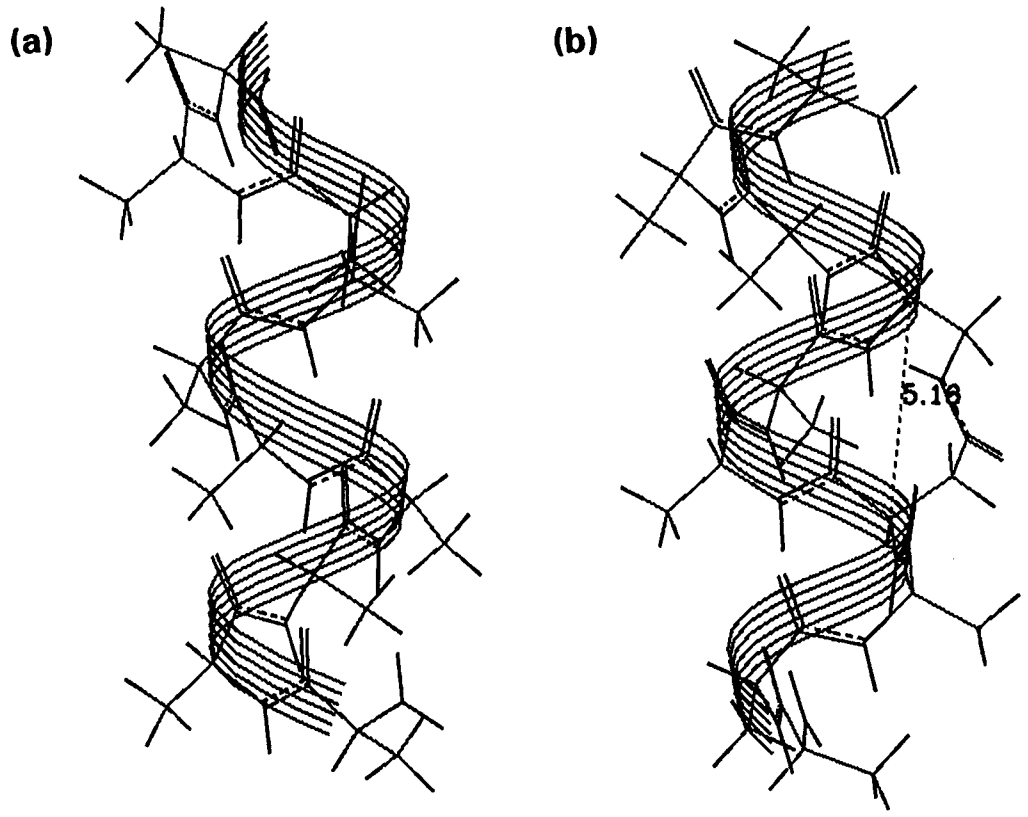


Figure 1.2 Proposed helical structure of polyalanine (1-10) containing a cyclic constraint
(a) polyalanine (1-10) in a helical conformation
(b) a cyclic polyalanine analogue in a helical conformation with residues 4 and 7 replaced with D-Asp and Dap, respectively

helix is 5.19 [Aldrich, unpublished result] to 5.23 Å [Miranda et al., 1994], and the distance for that of D-Asp^{i} - Dap^{i+3} is 5.16 [Aldrich, unpublished result] to 5.12 Å [Marinda et al., 1994].

1.4.3 Design of Dyn A analogues

Dyn A-(1-13)NH_2 was utilized as the parent peptide because it has essentially the same biological activity as the full peptide Dyn A-(1-17), and it is the Dyn A fragment that was studied by Schwyzer. The C-terminal amide was introduced to increase stability to carboxypeptidase [Leslie, 1987]. The positions for D-Asp^{i} and Dap^{i+3} were selected which are not critical residues for opioid receptor recognition. Hence, four series of cyclic Dyn A-(1-13)NH_2 were prepared involving cyclization between residues 2 and 5, 3 and 6, 5 and 8, or 6 and 9 (see Figure 1.1). The effect of ring sizes was also examined by replacing Dap with Dab (α,γ-diaminobutyric acid) or Orn at residue $i+3$.

CHAPTER 2
LITERATURE REVIEW: OPIOIDS

2.1 History

Latex of the opium poppy, *Papaver somniferum*, has long been known for its action as an euphoriant and a pain killer. Morphine, the major active ingredient in the latex, has remained one of the most effective and widely used pain-relieving drugs today. Unfortunately, morphine has just as much potential for abuse as opium and possesses several undesirable side-effects such as tolerance, physical dependence, respiratory depression, constipation and sedation [Brownstein, 1993; Jaffe and Martin, 1990].

The problem of addiction to these opiate alkaloids has stimulated a search for potent analgesics without abuse potential or other undesirable side-effects. Although this effort has not achieved its goal, valuable information obtained during this extensive search has revealed some insights into the action of these opiate alkaloids. In 1973, three independent groups reported almost simultaneously that there are stereospecific binding sites for opiates in the central nervous system [Pert and Snyder, 1973; Simon et al., 1973; and Terenius, 1973]. The levorotatory enantiomer of the opiate alkaloids is usually the active isomer, whereas the dextrorotatory one is either much less active or inactive. The presence of very specific receptors in animals for a substance of plant origin was puzzling until Kosterlitz and his colleages [Kosterlitz and Waterfield, 1975] observed that brain extracts contain a factor with ability to inhibit acetylcholine release from nerves innervating the guinea pig ileum (GPI). The

substances were isolated and identified as the pentapeptides, Tyr-Gly-Gly-Phe-Met (Met-enkephalin) and Tyr-Gly-Gly-Phe-Leu (Leu-enkephalin) [Hughes et al., 1975]. Since then a number of peptides with opioid activity have been found.

A major breakthrough came in 1976 when Martin et al. [1976] provided evidence for the existence of three opioid receptor types, based on the analysis of the neurophysiological and behavioral properties of several opiate compounds in the chronic spinal dog (Table 2.1). These receptors were named after the prototype drugs used in the study: mu (μ) for morphine, kappa (κ) for ketocyclazocine and sigma (σ) for SKF 10,047 or *N*-allylnormetazocine. A year later, Kosterlitz and his colleagues [Lord, 1977] proposed a fourth type of opioid receptors, the delta (δ) receptor, for the enkephalins. The σ receptor is now generally considered not to be an opioid receptor because opioid antagonists do not reverse effects at this receptor [Zukin and Zukin, 1981].

More opioid receptor types and subtypes have been postulated, but their existence and physiological roles are not clear and hence they have not been widely accepted [Fries, 1991]. Recently, the three major opioid receptor types μ, κ and δ were successfully isolated and purified from several tissues, and these receptors cloned and expressed from cDNA libraries (see more details in section 2.4).

Table 2.1 Pharmacological effects of μ, κ and σ opioid agonists in the chronic spinal dog

	Morphine	Ketocyclazocine	NANM
Pupil	Miosis	Miosis	Mydriasis
Respiratory rate	Stimulation, then depression	No change	Stimulation
Heart rate	Bradycardia	No change	Tachycardia
Body temperature	Hypothermia	No change	No change
Affect	Indifference	Sedation	Delirium
Nociceptive reflexes:			
flexor	Decrease	Decrease	Modest decrease
skin-twitch	Decrease	No effect	No effect

Note: NANM = SKF 10,047 or N-allylnormetazocine.

Source: Martin, W.R.; Eades, C.G.; Thompson, J.A.; Huppler, R.E.; Gilbert, P.E. The Effects of Morphine and Nalorphine Like Drugs in the Nondependent and Morphine-Dependent Chronic Spinal Dog. *J. Pharmacol. Exp. Ther.* **1976**, *197*, 517-532.

2.2 Endogenous opioid peptides

Opioid peptides have been found in the central and peripheral nervous systems of both vertebrate and invertebrate animals [König, 1993]. More than 40 opioid peptides have been isolated and characterized. In addition to these endogenous peptides, some peptides from food digestion can exhibit opioid activity. These peptides are called "exorphins" because of their origins [König, 1993].

The mammalian opioid peptides vary considerably, ranging from 5 to 31 amino acid residues (Figure 2.1). Nevertheless, the N-terminal sequence of these peptides invariably consists of the sequence Tyr-Gly-Gly-Phe, which is thought to be responsible for their opioid activity. All of the mammalian opioid peptides known are derived from three large precursor proteins, proopiomelanocortin (POMC), proenkephalin and prodynorphin (or proenkephalin B) [Numa, 1984]. Each protein is coded by a separate gene. They have nearly identical molecular sizes, consisting of about 260 amino acids with a signal peptide.

The protein POMC was the first opioid peptide precursor to be sequenced [Nakanishi et al., 1979]. It exists mainly in the pituitary gland and the central nervous system. In addition to being the precursor of the endogenous opioid peptide β-endorphin, POMC also gives rise to adrenocorticotropin (ACTH) and a family of melanocyte-stimulating hormones (Figure 2.2). Proenkephalin was first discovered in bovine adrenal medulla [Noda et al., 1982]. It contains one copy of Leu-enkephalin, four copies of Met-enkephalin and two copies of C-terminal extended Met-enkephalins (Figure 2.2). Prodynorphin (or proenkephalin B) was the last opioid peptide

Precursor	Peptides	Structures
Pro-opio melanocortin	α-endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Glu-Thr-Pro-Leu-Val-Thr_{16}
	β-endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Glu-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-His-Lys-Lys-Gly-Gly-Glu_{31}
	γ-endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Glu-Thr-Pro-Leu-Val-Thr-Leu_{17}
Proenkephalin	Met-enkephalin	Tyr-Gly-Gly-Phe-Met_5
	Leu-enkephalin	Tyr-Gly-Gly-Phe-Leu_5
	Met-enkephalin-Arg^6-Phe^7	Tyr-Gly-Gly-Phe-Met-Arg-Phe_7
	Met-enkephalin-Arg^6-Gly^7-Leu^8	Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu_8
Prodynorphin	α-neo-endorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys_{10}
	β-neo-endorphin	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro_9
	Dynorphin A	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln_{17}
	Dynorphin A-(1-8)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile_8
	Dynorphin B (Rimorphin)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gly-Phe-Lys-Val-Val-Thr_{13}

Figure 2.1 Mammalian opioid peptides

Source: Simon, E.J. Opioid Receptors and Endogenous Opioid Peptides. *Med. Res. Reviews* **1991**, *11*, 357-374.

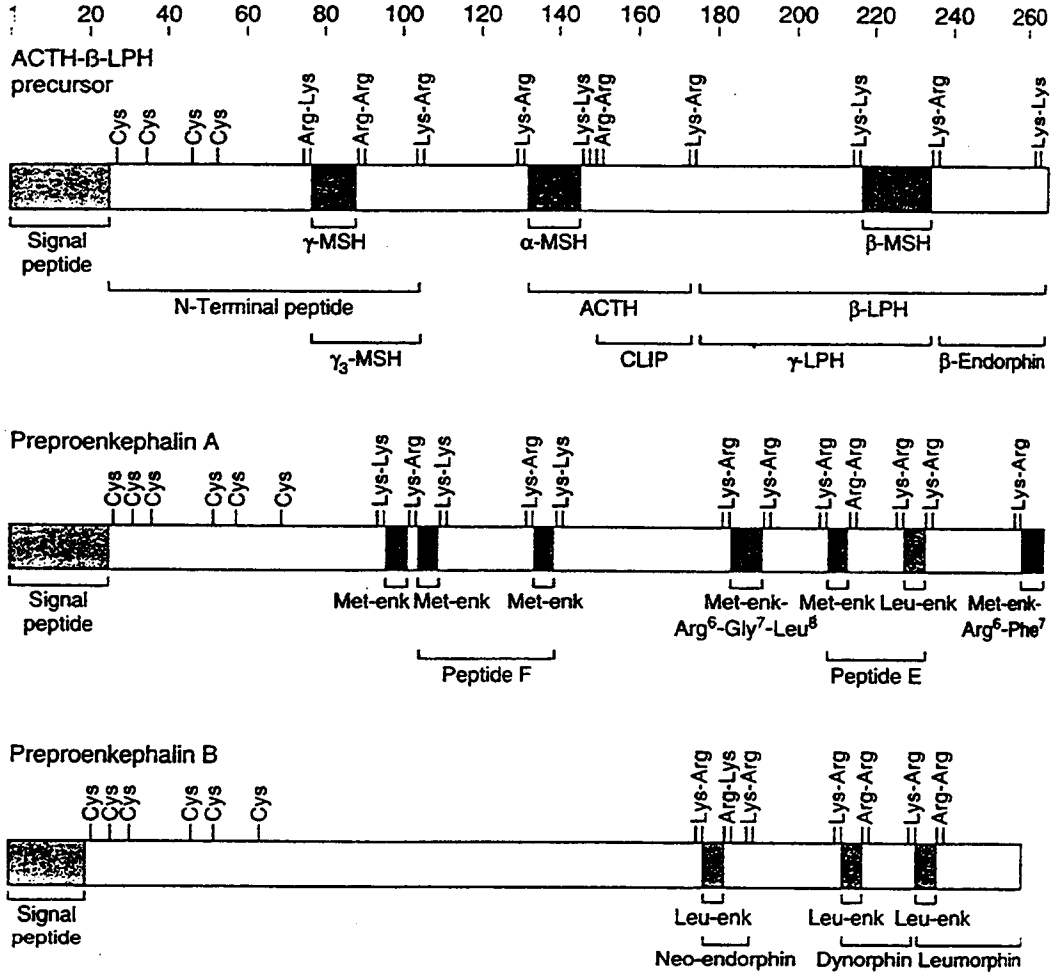


Figure 2.2 The precursor proteins for mammalian opioid peptides

Source: Simon, E.J.; Hiller, J.M. Opioid Peptides and Opioid Receptors. In *Basic Neurochemistry: Molecular, Cellular, and Medical Aspects*, 5th ed.; Siegel, G.J.et al. Eds.; Raven Press, Ltd., New York: 1994, pp 321-339.

precursor to be characterized [Fischli et al., 1982]. It was isolated from various mammalian tissues, including brain and spinal cord, pituitary, adrenal, and reproductive organs. The opioid peptides derived from this protein are dynorphin A, dynorphin B (rimorphin), and α- and β-neo-endorphins (Figure 2.2).

The enkephalins have been postulated as putative endogenous ligands for δ receptors [Lord et al., 1977], while the dynorphins are proposed to be endogenous ligands for κ receptors [Chavkin et al. 1982]. The endogenous ligand for μ receptors is still unclear. A number of candidates have been advanced as possible endogenous ligands for this receptor type including β-endorphin, morphine, as well as enkephalins for which the μ receptor could be an isoreceptor [Simon, 1991]. In addition, morphine and its biosynthesis has been observed in mammalian brain [Weitz et al., 1986; Donnerer et al., 1986], raising a question about endogenous actions for this ligand.

Besides the mammalian endogenous peptides, the dermorphins and deltorphins found in the skin of the South American frogs *Phyllomedusa spp.* also exhibit opioid activity [Montecucchi et al., 1981 and Erspamer et al., 1989]. They are heptapeptides containing a D-amino acid at position 2 (Figure 2.3). The deltorphins are the most selective natural ligands with highest affinity for δ-opioid binding sites, whereas the dermorphins are considered the most potent and selective natural ligands for μ-receptors in both peripheral and central nervous systems [Erspamer, 1992].

It has been reported that peptide fragments from enzymatic degradation of food can exhibit opioid activity [Brantl et al., 1981]. β-Casomorphins, isolated from the

Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2	Dermorphin
Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2	D-Met-Deltorphin (Dermenkephalin)
Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2	[D-Ala^2]-Deltorphin I
Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2	[D-Ala^2]-Deltorphin II

Figure 2.3 Opioid peptides from the amphibian skin

Source: Erspamer, V. The Opioid Peptides of the Amphibian Skin. *Int. J. Devl. Neuroscience* **1992**, *10*, 3-30.

milk protein casein, are considered to be μ receptor agonists. The *N*-terminal β-casomorphin tetrapeptide amide, morphiceptin, shows good opioid activity, and it is a highly μ selective ligand [Chang et al., 1981]. These peptides contain an identical N-terminal sequence Tyr-Pro-Phe-Pro (Figure 2.4). Other exogenous peptides reported with opioid activity are: gluten exorphin C from wheat gluten [Fukudome and Yoshikawa, 1993], α-casomorphins fron α-casein, and F12-5 from cobra poison [König, 1993].

2.3 Highly selective ligands for different opioid receptor types

The putative natural ligands of the different types of opioid receptors and some opiate alkaloids exhibit considerable affinity for more than one receptor type [Simon, 1991]. Hence, a number of synthetic opioid ligands have been designed such that these ligands exhibit some selectivity for one opioid receptor type. Table 2.2 shows ligands exhibiting some selectivity for one opioid receptor type and commonly used in studies of the endogenous opioid system. Some of these ligands are analogues of opioid peptides. The modified ligands based upon the enkephalin structure generally show high selectivity toward μ or δ opioid receptors, such as DAMGO ([D-Ala^2,MePhe^4-Gly-ol]enkephalin for μ receptors [Handa et al., 1981] and DPDPE (cyclo[D-Pen^2,D-Pen^5]enkephalin) for δ receptors [Mosberg et al., 1983]. Some analogues were developed from a peptide not related to enkephalins such as CTOP and CTAP [Kramer et al., 1989]. Both of these peptide analogues were derived from the peptide somatostatin and surprisingly exhibited antagonist activity at opioid receptors with high selectivity for μ receptors.

α-Casomorphin-7	Arg-Tyr-Leu-Gly-Tyr-Leu-Asp
β-Casomorphin-7	Tyr-Pro-Phe-Pro-Gly-Pro-Ile
Morphiceptin	Tyr-Pro-Phe-Pro-NH_2
F12-5	Val-Pro-Arg-Pro-Tyr
Gluten exorphin C	Tyr-Pro-Ile-Ser-Leu

Figure 2.4 Opioid exorphins

Source: König, W. Opioid Peptides. In *Peptide and Protein Hormones: Sructure, Regulation, Activity. A Reference Manual.* VCH: New York, 1993, pp 69-88.

Table 2.2 Ligands commonly used in studies of the endogenous opioid system

Compound	Type		Structure
	AG	ANT	
Morphine	μ		Alkaloid
Naloxone		μ, δ (κ)	Alkaloid, with *N*-allyl substituent on basic nitrogen atom
Naltrexone		μ, δ (κ)	Alkaloid, with cyclopropylmethyl substituent on basic nitrogen atom
Levorphanol	μ		Morphinan
Cyprodime		μ	Morphinan
DAGO	μ		Tyr-D-Ala-Gly-MePhe-Gly-ol, an enkephalin analog
Dermorphin	μ		Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2, a native peptide from frog (genus *Phyllomedusa*) skin
Morphiceptin	μ		Tyr-Pro-Phe-Pro-NH_2, a peptide derived from β-casein
CTOP		μ	D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2, cyclic octapeptide related to somatostatin
Leu-enkephalin	δ, μ		Tyr-Gly-Gly-Phe-Leu
DADLE	δ, μ		Tyr, D-Ala-Gly-Phe-D-Leu
DSLET	δ_2		Tyr-D-Ser-Gly-Gly-Phe-Leu-Thr
DPDPE	δ_1		[D-Pen^2, D-Pen^5]enkephalin, cyclic peptide
Deltorphin	δ_2		Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2, a native peptide from frog skin
Naltrindole		δ_2	6,7-Indole analog of naltrexone
ICI 174864		δ	*N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu
D-Prodyn	κ		Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-D-Pro-Lys
Ethylketocyclazocine	κ	μ, δ	Benzomorphan
Bremazocine	κ	μ, δ	Benzomorphan
U50,488H	κ		Benzeneacetamide
U69,593	κ_1		Benzeneacetamide
Nor-binaltorphimine		κ	Dimeric naltrexone derivative

(AG) Agonist; (ANT) Antagonist. Parentheses denote very low affinity.

Source: Simon, E.J.; Hiller, J.M. Opioid Peptides and Opioid Receptors. In *Basic Neurochemistry: Molecular, Cellular, and Medical Aspects*, 5th ed.; Siegel, G.J.et al. Eds.; Raven Press, Ltd., New York: 1994, pp 321-339.

Several ligands have been derived form small synthetic molecules which have a non-peptide structure, mostly based on the morphine structure (Figure 2.5) [Ree and Hunter, 1990]. Dihydromorphine is a μ-selective ligand with slightly increased potency compared to morphine, whereas EKC and bremazocine poorly distinguish between opioid receptor types even though they have frequently been used for characterization of κ binding sites. Introduction of an *N*-allyl or *N*-cyclopropylmethyl on the basic nitrogen atom of morphine commonly give compounds with opioid antagonist activity such as naloxone and naltrexone. Other synthetic compound series with opioid activity with distinct chemical structures from the morphine skeleton are arylacetamides, anilidopiperidines and benzodiazepines (Figure 2.5). The aryacetamides U69593 and U50488 are highly selective ligands for κ receptors. The benzodiazepine tifluadom also exhibits excellent binding affinity for κ over μ and δ receptors, whereas anilidopiperidine derivatives, such as sulfentanil, are highly selective for μ receptors.

2.4 Opioid receptors

Similar to their endogenous peptides, opioid receptors are found in the central nervous system and in a number of peripheral tissues [Simon, 1986a,b]. There are several regions in brain that contain μ receptors, and there is evidence of a very high proportion of this receptor type in rat [Chang et al., 1979], bovine [Ninkovic et al., 1981] and human thalamus [Bonnet et al., 1981], and in rabbit cerebellum [Meunier et al., 1983]. There are some brain regions which are enriched in δ receptors, but they also contain large amounts of other receptor types. The system known to be

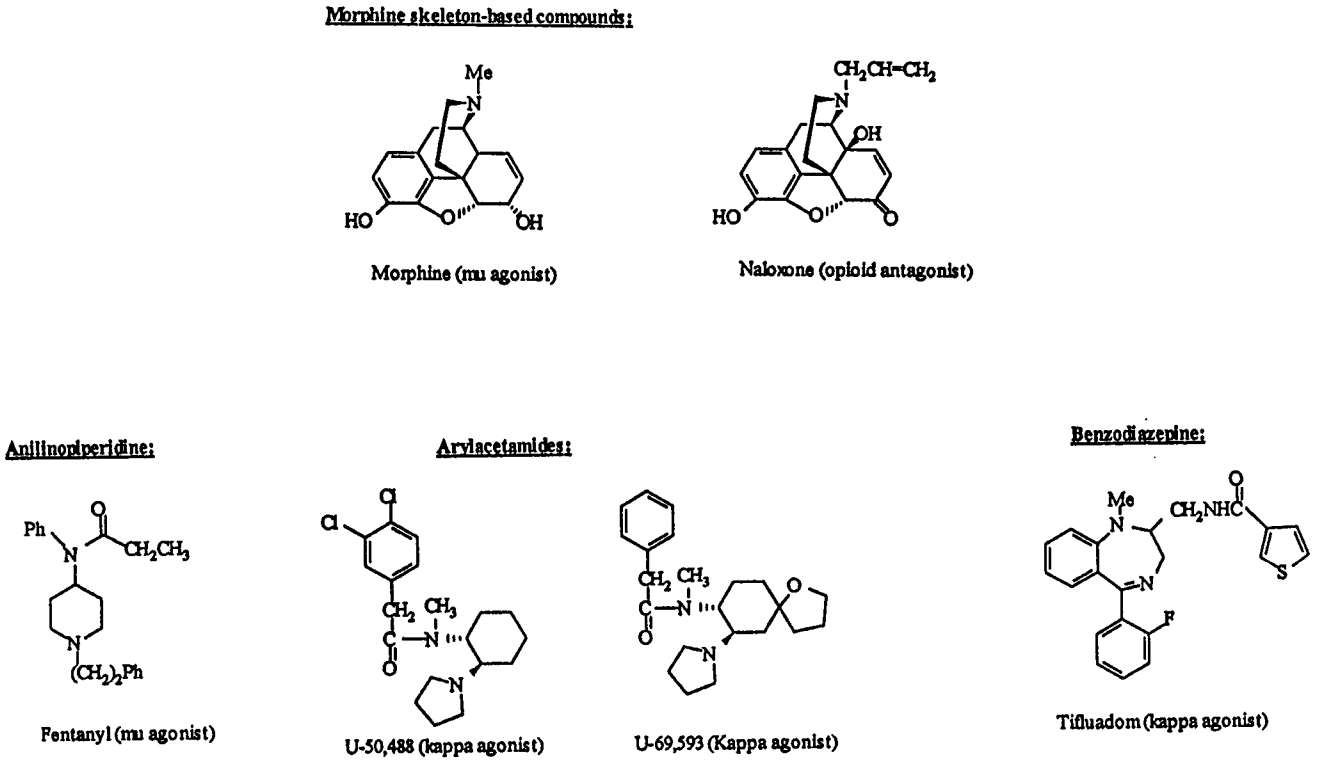


Figure 2.5 Structures of some non-peptide opioid ligands

Source: McKnight, A.T.; Rees, D.C. Opioid Receptors and their Ligands. *Neurotransmissions* **1991**, *7*, 1-6.

highly enriched in δ receptors are the neuroblastoma cells (N4TG1) and the neuroblastoma x glioma hybrid cells (NG-108-15) in culture [Chang and Cuatrecasas, 1979]. For κ receptors, there are several tissues reported to contain very high proportions of this receptor. These include human placenta [Valette et al., 1980], toad brain [Simon et al., 1982], and guinea pig cerebellum [Robson et al., 1984]. The opioid receptors in the guinea pig cerebellum are reported to be almost exclusively (85-100%) the κ receptor type.

The peripheral tissues used most extensively in biological assays are the mouse vas deferens (MVD) and the guinea pig ileum (GPI). Both tissues contain a heterogenous population of opioid receptors. Although the mouse vas deferens contains all three, δ, μ and κ, receptor types [Chang, 1984], the opioid activity observed results predominantly from interaction with δ receptors and less from interaction with μ and κ receptors [Leslie, 1987]. Recently, Cohen et al. [1994] have taken advantage of the existence of all three opioid receptors in the MVD to determine affinities of antagonists, particularly for κ receptors. In the guinea pig ileum, opioid activities are mediated through μ and κ receptors, the predominant receptors found in this tissue [Leslie, 1987]. Homogenous opioid receptor types can be found in other isolated tissue preparations, such as the hamster vas deferens (HVD) for δ receptors [McKnight et al., 1984] and the rabbit vas deferens (LVD) for κ receptors [Oka et al., 1980].

2.4.1 Isolation and purification of opioid receptors

Since their discovery, the isolation and purification of opioid receptors have been one major area of effort in opioid research. Recently, purifications to apparent homogeneity of the μ, δ and κ opioid binding proteins have been achieved (Table 2.3) [Barnard and Simon, 1993]. Most binding sites purified are inactive forms (arttributed to the absence of G-proteins) consisting of polypeptides with M_r ~ 60,000. The first receptor type to be separated in its active form was the κ receptor [Itzhak et al., 1984], but purification to homogeneity of active binding sites has been achieved for the μ receptor [Gioannini et al., 1985]. For δ receptors, the active binding sites have not yet been purified.

2.4.2. Molecular biology of opioid receptors

Recently, all three major opioid receptor types (μ, δ and κ) have been successfully cloned from cDNA libraries. Evans et al. [1992] and Kieffer et al. [1992] reported almost simultaneously that the δ receptors could be cloned from cDNA libraries of NG-108-15 cells. Using a similar procedure, Yasuda et al. [1993] identified two unique clones from mouse brain and showed that one of the cloned receptors was identical to the δ receptors cloned earlier from NG-108-15 cells and that the other clone could be κ opioid receptors. The μ opioid receptors were cloned from rat brain cDNA library [Chen et al., 1993a; Zastawny et al., 1994]. Cloned κ receptors were also identified from a rat brain cDNA library [Chen et al., 1993b] and from a guinea pig brain cDNA library [Xie et al, 1994]. The κ receptor cloned from guinea pig brain is 90% identical to the mouse and rat κ receptors. All three opioid

Table 2.3 Affinity purification of opioid-binding proteins from brain tissue

Tissue[a]	Receptor	Coupled ligand	M_r (Kilodaltons, K) of subunits present	Yield (%)	Purification[b] (fold, approx.)
Rat	μ	BAM	43 K, 35 K, 23 K	ns	2000
Rat	μ or δ	DALE	62 K	ns	450
Bovine	μ	Hybromet	94 K	0.6	500
NG108-15	δ	AntiFIT	58 K	3	30,000
Bovine	μ	β-naltrexethylene diamine	65 K	5.8	65,000–75,000
Rat	μ	6-Succinylmorphine	58 K	6	68,000
Rat	μ	DALECK	62 K, 54 K	10	20,000–30,000
Rat	μ	GANC	62 K	8	60,000–70,000
Rat	μ	6-Succinylmorphine	58 K	ns	60,000[c]
Frog	κ	DALE	65 K, 58 K	0.07	4300
Frog	κ	Dynorphin(1–10)	65 K	8	19,600[c]
Guinea pig	κ	[Ala[11]]dynorphin(1–11)	62 K	6	15,000–20,000[c]

AntiFIT: antibody to the fentanyl group; BAM: 14-β-bromo-acetamido morphine; DALE: [D[Ala^2,L-Leu^5]enkephalin; DALECK: DALE-chloromethyl ketone; GANC: 14-β[(-glycyl)-amido(N-cyclopropymethyl)] norcodeinone; Hybromet: 7α-(1R)-hydroxy-1-methyl-3-p[4-(3'bromomercury-2'-methoxy-propoxy)phenyl]-propyl-6,14-endoethenotetrahydro-thebaine; ns, yield not stated.

[a]All are brain membranes, except the NG108-15 cultured cell line.

[b]Fold purification (relative to the starting extract) and yield are obtained after removal of ligand, and in some cases increased purification, by additional steps (e.g. lectin or hydroxyapatite chromatography or gel filtration).

[c]Fold purification is given, when the receptors are reconstituted into lipid micelles, with or without G-proteins (see text).

Source: Barnard, E.A.; Simon, J. Opioid Receptors. In *The New Comprehensive Biochemistry Vol. 24: Neurotransmitter Receptors*, Hucho, F. Ed.; Elsevier: New York, 1993, pp 297-323.

receptor types cloned from different sources show a high degree of amino acid sequence similarity (Figure 2.6). This similarity was also found between somatostatin and opioid receptors, particulary κ receptors [Yasuda et al., 1993; Chen et al., 1993b].

All of the cloned opioid receptors were found to associate with pertussis-toxin-sensitive G proteins and mediate agonist inhibition of adenylate cyclase activity [Uhl et al., 1994]. Opioid receptors are postulated to be a sub-family of the G-protein-coupled neuropeptide receptors which are thought to contain seven hydrophobic transmembrane domains. The comparison of the amino acid sequence of the three cloned opioid receptors (Table 2.4) shows that the sequences of membrane spanning segments 2, 3 and 7 are highly conserved, whereas the sequences of segments 1, 4 and 5 are more divergent. The sequences of the intracellular loops are also highly conserved, especially the second intracellular loop, which is reported to be one of the G protein binding domains for other neuropeptide receptors. The amino and carboxyl termini of the opioid receptors differ considerably in amino acid sequence and in size. The extracellular amino terminus and the second and third extracellular loops have been hypothesized to constitute the ligand-binding domains of the different opioid receptors [Uhl et al., 1994].

2.4.3 Subtypes of opioid receptor types

Opioid receptor subtypes have been characterized based upon differentiaton in ligand binding studies and pharmacological experiments. The κ receptor type was first subclassified into two subtypes, κ_1 and κ_2, in the early 1980's [Pfeiffer et al.,

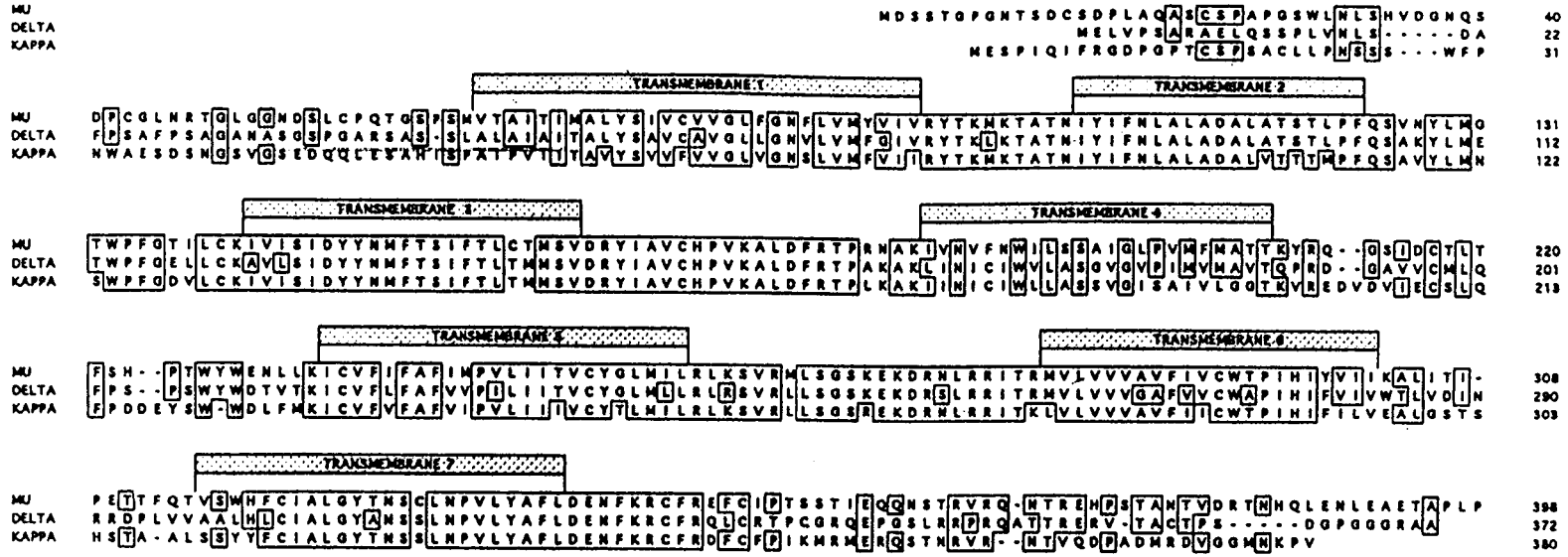


Figure 2.6 Alignment of the primary sequences for the opioid receptros (rat μ, mouse δ and mouse κ), using the single amino acid code. Box regions indicate conserved amino acids between the receptors μ [Zastawny et al., 1994], δ [Evans et al. 1992] and κ [Yasuda et al., 1993].

Source: Zastawny, R.L.; George, S.R.; Nguyen, T.; Cheng, R.; Tsatsos, J.; Briones-Urbina, R.; O'Dowd, B.F. Cloning, Characterization, and Distribution of a μ-Opioid Receptor in Rat Brain. *J. Neurochem.* **1994**, 62, *2099-2105.*

Table 2.4 Amino acid similarity profile of the κ, μ and δ opioid receptors and the somatostatin receptor

Structural region	Amino acid similarity		
	κ/μ	κ/δ	κ/SOM
Extracellular regions			
N-terminus	6/63 (10%)	5/53 (9%)	4/47 (9%)
First loop	12/18 (67%)	13/18 (72%)	5/18 (28%)
Second loop	12/28 (43%)	11/28 (39%)	5/29 (17%)
Third loop	5/14 (36%)	2/14 (14%)	1/14 (7%)
Transmembrane domains			
TM1	14/22 (64%)	13/22 (59%)	13/22 (59%)
TM2	19/22 (86%)	19/22 (86%)	12/22 (55%)
TM3	20/22 (91%)	20/22 (91%)	12/22 (55%)
TM4	7/22 (32%)	12/22 (55%)	5/22 (23%)
TM5	16/22 (73%)	16/22 (73%)	8/22 (36%)
TM6	16/22 (73%)	14/22 (64%)	13/22 (59%)
TM7	19/22 (86%)	18/22 (82%)	11/22 (50%)
Cytoplasmic regions			
First loop	7/7 (100%)	6/7 (86%)	6/7 (86%)
Second loop	20/22 (91%)	21/22 (95%)	12/22 (55%)
Third loop	21/24 (88%)	20/24 (83%)	5/24 (21%)
C-terminus (before palm.)	11/12 (92%)	11/12 (92%)	5/12 (42%)
C-terminus (after palm.)	7/35 (20%)	5/35 (14%)	1/35 (3%)

The similarity value is calculated for each region, with the numerator indicating the number of amino acids which are identical between the two sequences in the region and the denominator indicating the length of overlapping sequence in that region. The percentage similarity is shown in parentheses. The conserved palmitoylated (abbreviated as "palm.") cysteine residue divides the C-terminus into two regions, the N-terminal portion (before palm.) and the C-terminal portion (after palm.). Abbreviation: SOM, somatostatin receptor.

Source: Chen, Y.; Mestek, A.; Liu, J.; Yu, L. Molecular Cloning of a Rat κ Opioid Receptor Reveals Sequence Similarities to the μ and δ Opioid Receptors. *Biochem. J.* **1993**, *295*, 625-628.

1981; Attali et al., 1982]. The κ_1 sites differ from the κ_2 sites in that the κ_1 sites bind with high affinity to the arylacetamide U-69593, whereas the κ_2 sites have low affinity for this compound. The benzomorphans EKC and bremazocine appear not to be selective for either κ_1 or κ_2 subtypes. Dynorphins also bind to both subtypes, with a preference toward the κ_1 subtype. An additional subtype, κ_3, was proposed based upon studies with the compound [^{3}H]naloxone benzoylhydrazone [Clark et al., 1989]. It has also been proposed that the κ_3 subtype behaves like an isoform of μ receptors [Wollemann et al., 1992; Bardnard and Simon, 1993].

The μ receptors have been subdivided by Pasternak and Wood [1986] into μ_1 and μ_2 subtypes. The postulated μ_1 sites bind both morphine and enkephalins with high affinity, while the μ_2 are the "classical" μ receptors which have a significantly lower affinity for enkephalins than for morphine. The μ_1 sites differ from δ receptors in that the δ receptors have low affinity for morphine and a much higher affinity for DPDPE, a δ selective ligand. The excistence of μ_1 subtypes, however, has not been well accepted because a very low density of this subtype was found in rich sources of μ receptor sites (i.e. thalamus and rat brain) [Barnard and Simon, 1993] and because there is not yet a highly specific ligand for differentiating μ_1 from μ_2 sites [Goldstein and Naidu, 1989].

The δ receptors in the central nervous system have been proposed to consist of two subtypes, δ_1 and δ_2 [Traynor and Elliott, 1993]. DPDPE and deltorphin I are much more potent at δ_1 receptors, whereas δ_2 sites are preferred by DSLET and [D-

Ala^2]deltorphin II. The existence of these two separate receptor subtypes was further supported by lack of cross-tolerance *in vivo* between the two types of δ agonists.

Recently, Raynor and co-workers [1994] have characterized the cloned receptors using a number of diverse opioid ligands (Table 2.5). The results suggested that the cloned κ receptors had pharmacological characteristics similar to those of the endogenously expressed κ_1 receptors, and that the cloned δ receptor displayed a pharmacological profile consistent with that of δ_2 receptors.

2.5 Pharmacologic methods for identification of opioid receptors

Due to the multiplicity and heterogeniety of the opioid receptors, the determination of which opioid receptor types are involved in the observed activity of tested compounds is therefore important, and it is necessary to determine their selectivity. Two approaches generally utilized to evaluate opioid receptor selectivity are binding selectivity and pharmacological selectivity [James and Goldstein, 1984; Goldstein and James, 1984]. Binding selectivity is the ratio of affinities for a given opioid ligand at several types of binding sites [James and Goldstein, 1984]. The affinity of an opioid ligand is usually determined by competition binding assays, and expressed as an inhibition constant (K_i). The K_i values can be calculated from IC_{50} values (concentration of a ligand producing 50% inhibition) using the Cheng and Prusoff equation [Cheng and Prusoff, 1973]. A highly selective radioligand is normally used for competing at a particular binding site [Leslie, 1987]. A tissue preparation with a high population of one binding site can be used to provide more specific binding if a selective radioligand is not available. Since binding affinity does

Table 2.5 Binding potencies (K_i) of ligands for the cloned κ, δ and μ opioid receptors

	κ Receptor, [^{3}H]U-69,593	δ Receptor, [^{3}H]naltrindole	μ Receptor, [^{3}H]DAMGO
		nM	
Nonselective compounds			
Dynorphin A	0.5	>1000	32
Leu-enkephalin	>1000	4.0	3.4
Met-enkephalin	>1000	1.7	0.65
β-Endorphin	52	1.0	1.0
Des-Tyr^1-β-endorphin	>1000	>1000	>1000
(−)-Naloxone	2.3	17	0.93
(+)-Naloxone	>1000	>1000	>1000
Levorphanol	6.5	5.0	0.086
Dextrorphan	>1000	>1000	>1000
(±)-Bremazocine	0.089	2.3	0.75
Ethylketocyclazocine	0.40	101	3.1
Etorphine	0.13	1.4	0.23
Pentazocine	7.2	31	5.7
Diprenorphine	0.017	0.23	0.072
β-CNA[a]	0.083	115	0.90
β-FNA	2.8	48	0.33
Naltrexone	3.9	149	1.0
Nalbuphine	3.9	>1000	11
Nalorphine	1.1	148	0.97
μ-Selective compounds			
CTOP	>1000	>1000	0.18
Dermorphin	>1000	>1000	0.33
Methadone	>1000	>1000	0.72
DAMGO	>1000	>1000	2.0
PLO17	>1000	>1000	30
Morphiceptin	>1000	>1000	56
Codeine	>1000	>1000	79
Fentanyl	255	>1000	0.39
Sufentanil	75	50	0.15
Lofentanil	5.9	5.5	0.68
Naloxonazine	11	8.6	0.054
Morphine	538	>1000	14
κ-Selective compounds			
Norbinaltorphimine	0.027	65	2.2
Spiradoline	0.036	>1000	21
U-50,488	0.12	>1000	>1000
U-69,593	0.59	>1000	>1000
ICI 204,488	0.71	>1000	>1000
δ-Selective compounds			
DPDPE	>1000	14	>1000
D-Ala^2-deltorphin II	>1000	3.3	>1000
DSLET	>1000	4.8	39
BW 3734	17	0.013	26
DADL	>1000	0.74	16
SIOM	>1000	1.7	33
Naltrindole	66	0.02	64
NTB	13	0.013	12
BNTX	55	0.66	18

[a] β-CNA, β-chloronaltrexamine; β-FNA, β-funaltrexamine; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; SIOM, 7-spiroindinooxymorphone.

Source: Raynor, K.; Kong, H.; Chen, Y.; Yasuda, K.; Yu, L.; Bell, G.I.; Reisine, T. Pharmacological Characterization of the Cloned κ-, δ-, and μ-Opioid Receptors. *Mol. Pharmacol.* **1994**, *45*, 330-334.

not depend on number of receptors and is not likely to change from tissue to tissue as long as conditions for the binding assay (i.e. buffer, temperature, etc.) are kept the same, general statements can be made about binding selectivity of a given ligand. The binding affinity of a ligand, however, does not provide information about whether it is an agonist or antagonist. Opioid receptors are reported to be sensitive to metal ions, especially sodium so that sodium reduces agonist affinity while it increases or does not affect antagonist affinity [Pert and Snyder, 1974]. Therefore, sodium effects measured by the "sodium index" can be utilized to help distinguish pure agonist, pure antagonist, and mixed agonist-antagonist actions of opioid ligands [Simon, 1986a].

Bioassays are commonly used to determine opioid activity *in vitro* [Leslie,1987]. Opioid compounds act through opioid receptors in the GPI or MVD preparations to inhibit the electrically stimulated contraction of the muscle. For a particular compound, the ratio of potencies at the different receptor types indicates its pharmacological selectivity [James and Goldstein, 1984]. It is difficult to make general statements about pharmacological selectivity because the observed potency is dependent upon the compound's affinity for a given receptor type, the intrinsic activity or efficacy of the agonist, and the number of receptors in the tissue.

There are two methods commonly used to determine which opioid receptor type in the GPI and MVD preparations are responsible for the opioid activity of an opioid ligand. Generally, the antagonist dissociation constant (K_e) for naloxone is used to differentiate the opioid activities mediated by μ, κ or δ receptors.

Determining the K_e for naloxone antagonism of an opioid ligand involves evaluation of ED_{50}'s (effective dose at 50% response) in the presence and absence of a single dose of naloxone. This method has the advantage of being fast and easy, but the accuracy and reliability are dependent upon the assumptions of competitive kinetics of the antagonist and the homogeneity of the receptor population. In GPI and MVD tissues, the K_e value for naloxone antagonism of μ opioid effects is 2 to 3 nM, while that for antagonism of responses mediated by κ or δ receptors is 20-30 nM [Leslie, 1987]. The antagonist pA_2 value has been considered to be a more definitive method for receptor classification. It provides the affinity of an antagonist for a receptor, and is determined by interception on the abscissa of the Schild plot [Arunlakshana and Schild, 1959]. The pA_2 value is equal $-\log[K_e]$ when the plot is linear with a slope of unity over a wide range of antagonist concentrations. The pA_2 values of naloxone and other opioid antagonists for each opioid receptor type are listed in Table 2.6.

2.6 Dynorphin A

Dynorphin A is a 17-mer peptide which was first isolated from porcine pituitary gland [Cox et al., 1975; Goldstein et al., 1981]. This peptide was named "dynorphin" (*dyn-* from the Greek *dynamis* signifying strength or power) because of its extraordinary potency in the GPI assay [Goldstein et al., 1979]. It also shows agonism in mouse vas deferens and in rabbit vas deferens [Goldstein et al., 1979; James, 1986]. Dyn A is postulated to be an endogenous ligand for κ receptors [Chavkin et al. 1982], but it also binds to μ and δ receptors with considerable affinity. Results from measurements of naloxone K_e values, selective alkylation or protection

Table 2.6 Antagonistic potencies of naloxone and naltrexone against various agonists

Preparation	Agonist	Naloxone		Naltrexone	
		pA_2 ± S.E.	Slope ± S.E.	pA_2 ± S.E.	Slope ± S.E.
Guinea pig ileum	Morphine	8.54 ± 0.06 (6)	-1.04 ± 0.04		
		8.37 ± 0.02 (4)	-1.19 ± 0.10	9.08 ± 0.06 (4)	-0.98 ± 0.05
		8.50 ± 0.06 (6)	-1.06 ± 0.05		
	Ethylketazocine	7.88 ± 0.10 (4)	-0.91 ± 0.03	8.09 ± 0.12 (4)	-1.09 ± 0.06
Mouse vas deferens	Morphine	8.66 ± 0.18 (3)	-0.92 ± 0.09	8.72 ± 0.04 (3)	-1.09 ± 0.06
	DADLE	7.39 ± 0.21 (4)	-1.02 ± 0.14	7.56 ± 0.24 (4)	-0.94 ± 0.15

Number of experimets are indicated in parentheses.

Source: Takemori, A.E.; Portoghese, P.S. Comparative Antagonism by Naltrexone and Naloxone of μ, κ, and δ Agonists. *Eur. J. Pharmacol.* **1984**, *104*, 101-104.

studies and cross-tolerance studies have confirmed that Dyn A acts at κ receptors in the GPI and MVD assays [James et al., 1984; James, 1986].

2.6.1. Conformations of dynorphin A

The bioactive conformation of dynorphin A has not been conclusively determined, and many possibilities have been proposed. Circular dichroism (CD) studies of Dyn A-(1-13) have generally led to the suggestion that Dyn A-(1-13) has an unordered structure in aqueous solutions [Maroun and Mattice, 1981; Renugopalakrishnan et al., 1988]. Studies using Raman spectroscopy and FT-IR suggest a mixture of extended and random structures [Renugopalakrishnan et al., 1988; Rapaka et al., 1987]. Based on 1D- and 2D-^{1}H NMR, Dyn A-(1-13) apparently assumes an extended structure in aqueous solution, whereas in DMSO the first five residues, Tyr-Gly-Gly-Phe-Leu, assume a type I β-turn [Renugopalakrishnan et al., 1990]. The extended structure of Dyn A-(1-13) in water is consistent with fluorescence studies reported by Schiller et al. [1983] for their modified analogue [Trp^4]Dyn A-(1-13).

Schwyzer [1991, 1992a] has proposed an affect of the lipid membrane on peptide conformation. He suggested that in aqueous solution random structures of peptides may become ordered and adopt molecular conformers with amphiphilic properties when they contact the polar-non-polar interface of a lipid membrane. For Dyn A-(1-13), the peptide could an adopt α-helical structure from residues 1 through 9 when it interacts with its target cell membranes (κ binding sites), see Figure 2.7 [Schwyzer, 1986]. This hypothesis has been supported by evidence from hydrophobic

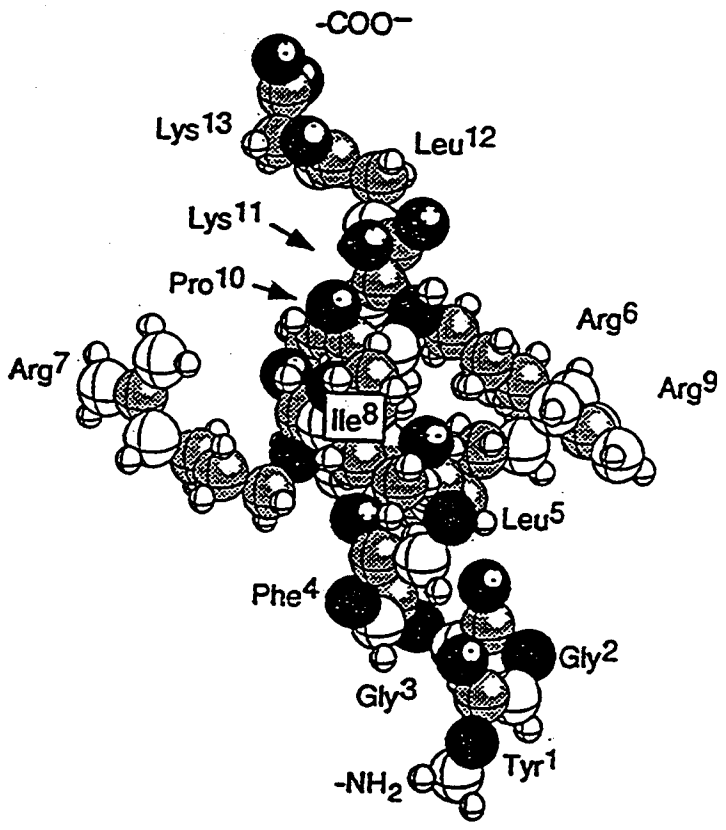


Figure 2.7 Possible conformation and orientation of Dyn A-(1-13) on a lipid membrane

Source: Schwyzer, R. Conformations and Orientations of Amphiphilic Peptides Induced by Artificial Lipid Membranes: Correlations with Biological Activity. *Biochem. Mol. Biol.* **1992**, *3*, 347-379.

labeling, CM (capacitance minimization), IRATR (polarized infrared in the attenuated total reflection mode), TRNOE (transferred nuclear Overhauser effect), energy estimations, and molecular modeling [Schwyzer, 1992b]. Recently, Kallick [1993] has also reported evidence of a helical structure of Dyn A in dodecylphosphocholine micelles using 2D-NMR.

An alternative secondary structure of Dyn A induced by a lipid membrane has also been proposed. A study using ^{1}H NMR and CD to determine the conformation of a model peptide for Dyn A-(1-17), Tyr-Gly-Gly-Phe-Leu-Lys-Lys-Val-Lys-Pro-Lys-Val-Lys-Val-Lys-Ser-Ser, suggested that an amphiphilic β-strand conformation may be adopted by residues 7 - 15 of the model peptide Dyn A on cell membranes [Vaughn and Taylor, 1989].

2.6.2 Structure-activity relationships

Similar to other opioid ligands, the key structures of Dyn A for opioid effects are the basic nitrogen and phenolic functionality of Tyr^1, and another aromatic ring at an appropriate position in space (Phe^4 for Dyn A) [Hansen and Morgan, 1984]. The predominant κ binding affinity of Dyn A is believed to be governed by its C-terminal amino acids [Chavkin and Goldstein, 1981]; the last four C-terminus residues of Dyn A (Trp-Asp-Asn-Gln) however, appear to be unnecessary for both κ receptor affinity and opioid activity [Corbett et al., 1982]. Based upon the similarities in sequence of opioid peptides, Goldstein [Chavkin and Goldstein, 1981] proposed a "message" sequence for Dyn A containing the N-terminal four residues and an "address" sequence containing amino acid residues 5 - 13 (Figure 2.8).

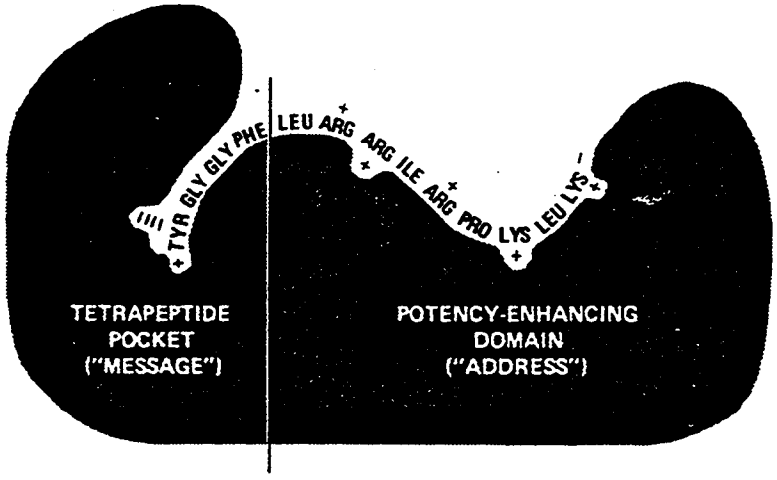


Figure 2.8 Diagram of the dynorphin receptor

Source: Chavkin, C.; Goldstein, A. Specific Receptor for the Opioid Peptide Dynorphin: Structure-Activity Relationships. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 6543-6547.

Dyn A contains five basic amino acid residues; two of which, Arg^{7} and Lys^{11} are critical for its potency and selectivity [Chavkin and Goldstein, 1981; Turcotte et al., 1984; Snyder et al., 1992]. A study using Lys(Ac) (i.e. the side chain amine of lysine was acetylated) to replace Arg^{6} indicated the importance of basicity at this position for κ selectivity [Snyder et al., 1992].

Other amino acid residues in the "address" sequence have also been studied. It was observed that Ile^8 and Arg^9 could be replaced with other amino acids without substantial loss of κ receptor selectivity or opioid activity [Snyder et al., 1992; Turcotte et al., 1984]. Substitution of lipophilic residues and certain D-amino acids at position 8 can increase κ receptor selectivty [Lamaire et al., 1986]. Similarly, substitution with D-Pro in position 10 of Dyn A-(1-11) or Dyn A-(1-13) improves κ selectivity considerably, and the high κ receptor affinities of these analogues are comparable with those of the unsubstituted peptides [Gairin et al, 1984].

Only a few modification has been studied for the "message" sequence. Substitution of Gly^2 with other amino acids generally reduced κ receptor selectivity and opioid activity [Story et al., 1992; Turcotte et al., 1984]. D-Amino acid substitution, however, is better tolerated than is substitution by the corresponding L-amino acids [Story et al., 1992]. Substituion of Gly^3 with D-Ala or L-Ala was recently reported to significantly improve κ selectivity with only a slight decrease in opioid activity in the GPI assay [Lung, et al., 1994].

Several conformational constraints have also been synthesized and studied for their opioid activity and opioid receptor affinity. More details on these constrained peptides will be discussed in chapters 6 and 7.

CHAPTER 3
LITERATURE REVIEW: PEPTIDE SYNTHESIS

3.1 Solid phase peptide synthesis

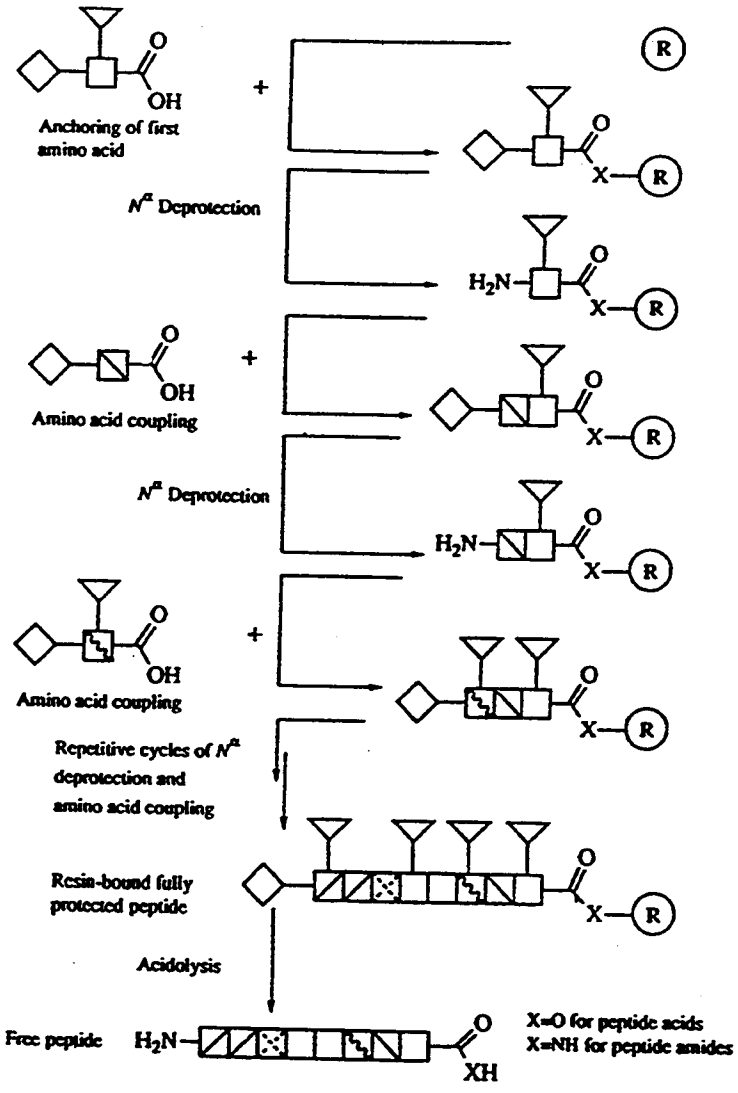
Chemical synthesis is probably the most practical way of providing useful quantities of peptides and allowing the simply synthetic variation of structure necessary for the development of peptides for pharmacological uses [Grant, 1992]. In principle, a peptide chain is formed by repetitively acylating a free amine of an amino acid with a carboxyl of another amino acid. For every coupling, purification and characterization of intermediate peptides obtained are required to ensure homogeneity of the final peptide product. The solution synthesis of a peptide is therefore time consuming, and can be challenging due to solubility problems of the growing peptide intermediates and physical losses during synthesis and purification.

The solid phase method of Merrifield [1963] represents an ingenious strategy for simplifying the incremental chain elongation of a peptide. The basic idea of the solid phase approach involves covalent attachment of the growing peptide chain to an insoluble polymeric support (resin) so that unreacted soluble reagents can be removed by simple filtration and washing without manipulative losses. Advantages of the solid phase method over solution phase syntheses are: (1) high speed, allowing six or more amino acid residues to be coupled per day; (2) no solubility problems of the kind that hamper solution synthesis; (3) ease and convenience of operation; and (4) mechanization and automation [Barany et al., 1987].

The scheme for the synthesis of a linear peptide using the solid phase strategy is shown in Figure 3.1. The amino acids assembled on a resin are usually added stepwise from the *C*-terminal end towards the *N*-terminal (*C* to *N* strategy) to minimize the chances of racemization which would be more pronounced if the synthesis were attempted in the opposite direction (*N* to *C* strategy) [Bodanszky, 1993]. The first amino acid of the sequence, protected at its N^{α}-terminus and at its side-chain (if necessary), is attached to a resin by either an ester linkage for a peptide acid or an amide bond for a peptide amide. Once the first amino acid is attached, the amino acid sequence of the peptide is built up on the resin by a series of N^{α}-deprotection and amino acid coupling steps. It is vital for solid phase synthesis that all reactions go to completion, since purification and characterization for each cycle of a solid phase synthesis is not feasible [Gross and Meienhofer, 1979]. When a desired sequence has been completely formed, the peptide is usually released from the resin simultaneously with deprotection of remaining protecting groups to afford a crude peptide.

3.2 Protecting groups

Three types of protection are required for solid phase peptide synthesis (Figure 3.2) [Stewart and Young, 1984]. The functional groups of amino acid side-chains must be protected with groups which are stable to the repetitive treatments necessary for removal of the N^{α}-amino protecting group on the growing peptide chain and for repeated amino acid coupling steps. Such side-chain protecting groups are usually called "semipermanent" protecting groups, and the protecting group of the N^{α} amino

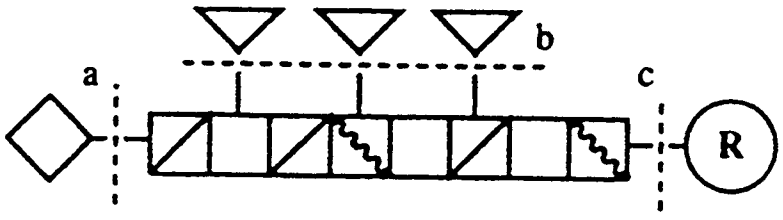


Note: Amino acids are represented by squares, the protected α-amino group by a diamond and the protected side-chain functional groups by triangles. The C-terminus of the peptide is bound to an insoluble solid support, designated R for resin.

Figure 3.1 Schematic representation of the synthesis of a linear peptide using solid phase strategy

Source: Lloyd-Williams, P.; Albericio, F.; Giralt, E. Convergent Solid-Phase Peptide Synthesis. *Tetrahedron* **1993**, *49*, 11065-11133.

functional groups is referred to as the "temporary" protecting group. The third type of protection is the peptide-resin anchorage which protects the *C*-terminus of the peptide throughout the various synthetic processes required to obtain the desired product.



a: N^{α}-Protecting group ("temporary")

b: Side-chain protecting groups ("semipermanent")

c: Resin-peptide anchorage

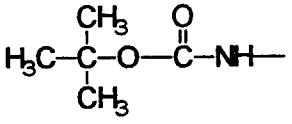
Figure 3.2 Protection scheme for solid phase peptide synthesis.

Source: Lloyd-Williams, P.; Albericio, F.; Giralt, E. Convergent Solid-Phase Peptide Synthesis. *Tetrahedron* **1993**, *49*, 11065-11133.

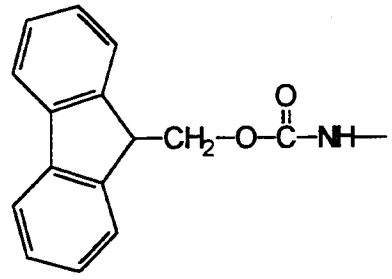
3.2.1 N^{α} amino protecting groups

The N^{α}-protecting groups commonly used are urethane derivatives (Figure 3.3). The N^{α}-*tert*-butyloxycarbonyl (Boc) group is stable to alkali or nucleophiles, and can be removed rapidly by inorganic or organic acids [Barany and Merifield, 1979]; the reagent most commonly used for deprotecting the Boc group is trifluoroacetic acid (TFA). The N^{α}-9-fluorenylmethoxycarbonyl (Fmoc) can be removed under basic conditions [Atherton and Sheppard, 1987]. The base commonly used is a secondary amine, usually piperidine, because dibenzofulvene (the product obtained from proton abstraction and subsequent β-elimination at the 9 position of the fluorene ring system) will be trapped by excess piperidine to from a stable and harmless adduct [Carpino and Han, 1972]. It has been reported that a non-nucleophilic base 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU; 2% in DMF) can be used as an alternative reagent for rapid and efficient Fmoc deprotection, particularly in continuous flow synthesis. This reagent, however, does not form dibenzofulvene adducts and hence must be washed rapidly from the peptide resin [Wade et al., 1991].

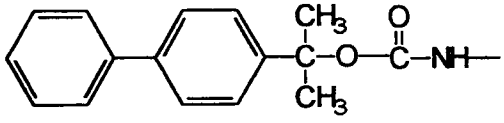
Other N^{α}-protecting groups which have been used in solid phase synthesis include the biphenylisopropyloxycarbonyl (Bpoc), the dimethoxyphenyl-propyloxycarbonyl (Ddz), the dithiasuccinoyl (Dts) and the nitropyridinesulfenyl (Npys) groups [Fields et al. 1992] (see Figure 3.3). The Bpoc and Ddz are highly acid labile protecting groups and can be removed in dilute TFA, while the Dts and Npys groups are removed by thiolysis.



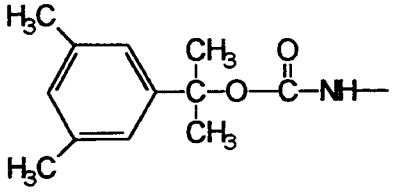
Boc



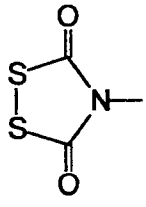
Fmoc



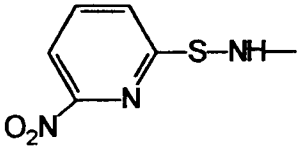
Bpoc



Ddz



Dts



Npys

Figure 3.3 The N^{α} protecting groups.

Source: Barany,G.; Jneib-Cordonier, N.; Mullen, D.G. Solid-Phase Peptide Synthesis: A Silver Anniversary Report. *Int. J. Peptide Protein Res.* **1987**, *30*, 705-739.

3.2.2 Side-chain protecting groups

Side-chain protecting groups are usually selected to be compatible with the N^{α}-amino functional groups and to minimize potential side reactions [Bodanszky and Martinez, 1983]. In the standard Merrifield system, the N^{α}-amino functional group is protected with Boc and the side-chain protecting groups commonly used are ether, ester or urethane derivatives based on benzyl alcohol. This protection scheme involves kinetic "fine-tunning", which is a reliance on quantitative rate differences in the acid susceptibility of different protecting groups [Barany et al. 1987].

Orthogonal protection is an alternative approach which involve classes of protecting groups that can be removed by different chemical mechanisms. In this case the N^{α}-amino protecting group is usually the Fmoc group and side-chain protection is typically provided by ether, ester, or urethane derivatives based on *tert*-butanol [Atherton and Sheppard, 1987].

Recently, allyl-based groups for side-chain protection have been introduced and gained acceptance for use in solid phase synthesis [Loffet and Zhang, 1993]. The allyl-based groups can be removed with a catalyst transition metal complex such as tetrakistriphenylphosphine palladium (0) ($(Ph_3P)_4Pd$) and a nucleophile (e.g. morpholine, dimedone, N-methylaniline or N,N'-dimethylbarbituric acid) [Kates et al. 1993]. Due to their distinct chemical reaction for deprotection, these allyl-based protecting groups provide a third dimension of orthogonality for the peptide synthetic scheme.

3.2.3 The solid support

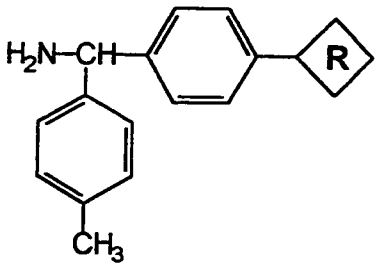
The resin support for solid phase peptide synthesis is often a polystyrene polymer cross-linked with 1% 1,3-divinylbenzene. The polystyrene resin swells adequately in apolar solvents such as dichloromethane (DCM). In contrast, the polystyrene resin has an inferior swelling property in a dipolar aprotic solvent such as dimethylformamide (DMF), but coupling rates in DMF are rapid enough to overcome this unfavorable effect [Atherton and Sheppard, 1989b]. Consequently, a polyamide resin (Pepsyn®) was developed so that both the insoluble support and the peptide backbone have comparable polarities and solvate well in DMF [Atherton and Sheppard, 1989c]. The polyethylene glycol-polystyrene graft support has recently been introduced for solid phase synthesis [Barany et al., 1992]. The use of this resin is becoming more popular because of its ability to swell in a wide range of solvents such as TFA, DMF, dimethylsulfoxide, acetonitrile and water [Auzanneau et al., 1995].

Almost all syntheses by the solid phase method generally start with the attachment of the C-terminal residue to the support, either directly or by means of a suitable handle. For Boc chemistry, the most common linkage to yield peptide acids is the benzyl ester [Stewart and Young, 1984]. This linkage is cleaved in a strong acid to liberate a free peptide. For Fmoc chemistry, peptide acids have been commonly generated using the 4-hydroxymethylphenoxy linker [Fields et al. 1992]. Peptides can be removed from this type of resin with 50-100% TFA.

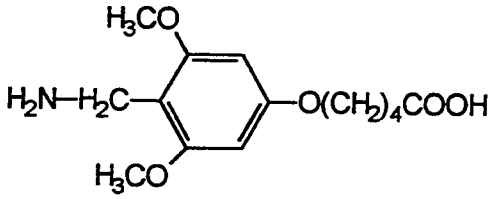
To obtain a peptide amide using Boc strategy, the anchoring linkages are generally benzhydrylamine derivatives which are removed by a strong acid and therefore compatible with the synthetic scheme of Boc chemistry. The 4-methylbenzhydrylamine resin (MBHA, see Figure 3. 4) is commonly used due to its greater acid lability than benzhydrylamine; free peptides can then be liberated with a strong acid in high yields [Stewart and Young, 1984]. An amino-methyl phenyl linker has also been modified with electron-donating methoxy groups to create the TFA-sensitive linkers for use in Fmoc chemistry, such as the PAL® resin which has 5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid linked on to an MBHA resin, see Figure 3.4 [Albericio et al., 1990].

3.3 Formation of peptide bonds

In solid phase synthesis, the formation of a peptide bond takes place within a well-solvated gel; therefore the apparent kinetic rate generally proceeds slower than the corresponding solution synthesis [Gross and Meienhofer, 1979]. A large excess of coupling reagents are routinely used to drive reactions to completion [Gross and Meienhofer, 1979]. Two approaches used extensively for peptide bond formation on a solid phase resin are *in situ* activation and the use of preactivated derivatives [Fields et al., 1992]. A characteristic of *in situ* coupling reagents is that they can be added to the mixture of carboxyl and amine components. Hence, activation and coupling proceed concurrently. In contrast, the use of preactivated derivatives requires two separate steps - the preparation of the activated carboxyl component and then the coupling reaction.



4-methylbenzhydrylamine resin



5-(4-aminomethyl-3,5-dimethoxy)valeric acid (PAL)

Figure 3.4 Resin linkers for peptide amides

Source: Fields, G.B.; Tian, Z.; Barany, G. Principles and Solid-Phase Peptide Synthesis. In *Synthetic Peptides, A User's Guide*. Grant, G.A., Ed.; W.H. Freeman and Company: New York, 1992, pp 77-183.

3.3.1 In situ activation

The type of *in situ* coupling reagent most extensively used is a carbodiimide (Figure 3.5), particularly *N,N'*-dicyclohexylcarbodiimide (DCC) and *N,N'*-diisopropylcarbodiimide (DIC) [Barany et al., 1987]. DIC is more convenient to use because the resultant urea byproduct is soluble in DCM, a solvent or co-solvent commonly used in coupling reactions. A side reaction associated with the use of the carbodiimides is the dehydration of the side-chain carboxamides of asparagine and glutamine to the corresponding nitriles [Ressler and Ratzkin, 1961]. Fortunately, this problem can be avoided by including the additive 1-hydroxybenzotriazole (HOBt; Figure 3.6) in the coupling reactions [Mojsov et al., 1980]. In addition, HOBt also accelerates the carbodiimide-mediated coupling, and suppresses racemization during the activation [König and Geiger, 1970].

The phosphonium reagents have become popular coupling reagents in solid phase synthesis because these reagents are highly efficient and provide exceptionally fast reaction rates; therefore a lower excess of carboxyl component can be used [Fields et al., 1992]. Introduced by Castro et al. [1975], benzotriazol-1-yl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP; Figure 3.5) has been used successfully in several peptide synthetic strategies including solution synthesis [Le-Nguyen et al., 1985], solid phase fragment condensation [Rivaille et al. 1980], solid phase synthesis [Le-Nguyen et al., 1987; Gausepohl, et al., 1989; Fournier et al., 1988; Fournier et al., 1989] and lactam formation [Felix et al., 1988b]. Although BOP is well recognized as an excellent coupling reagent, manufacturing or

utilizing BOP has involved the use or formation of a carcinogenic compound hexamethyl-phosphoric triamide (HMPA). A modified BOP reagent, benzotriazol-1-yl-oxy-trispyrrolidinophosphonium hexafluorophosphate (PyBOP; Figure 3.5), was therefore developed to avoid this hazard [Coste et al., 1990, Coste et al., 1992]. PyBOP is also superior to BOP in that it gives faster peptide bond formation and less epimerized byproduct [Coste, 1990]. Recently, Coste et al. [1994] have reported the application of novel phosphonium reagents containing halogen elements. Both PyBroP (bromotripyrrolidinophosphonium hexafluorphosphate, Figure 3.5) and PyCloP (chlorotripyrrolidinophosphonium hexafluorphosphate, Figure 3.5) are highly efficient reagents for coupling hindered N-methylated amino acids; these reactions are often difficult, and standard coupling reagents are often inefficient at driving these reactions to completion.

The replacement of the phosphorous atom in the phosphonium reagent by a carbon atom affords a new type of coupling reagent, the uronium reagent. HBTU (*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, Figure 3.5) was first employed for peptide synthesis by Dourtoglou et al. [1978]. It became well-known when Knorr and his colleage [1989] introduced a convenient synthetic route using a one-pot procedure and then synthesized more uronium derivatives. HBTU and TBTU (*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate, Figure 3.5) exhibited faster reaction rates and racemization comparable to BOP [Dourtoglou and Gross, 1984; Knorr et al., 1989]. *In situ* activations with excess HBTU and TBTU, however, can cap free amino functional groups giving a peptide byproduct

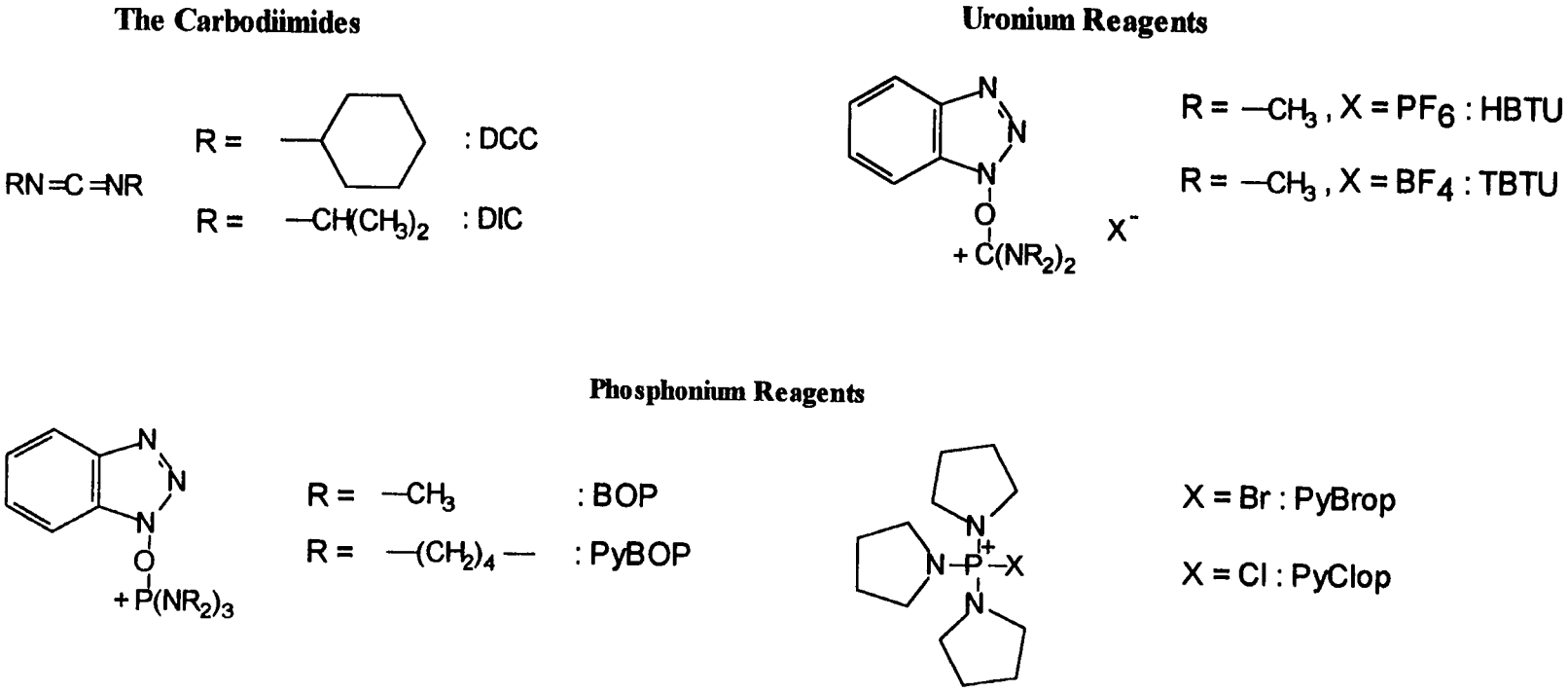
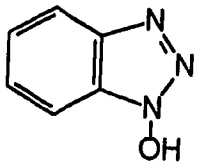


Figure 3.5 ***In Situ*** **Coupling Reagents**

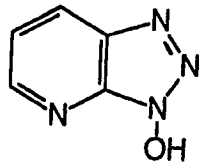
Sources: Fields, G.B.; Tian, Z.; Barany, G. Principles and Solid-Phase Peptide Synthesis. In *Synthetic Peptides, A User's Guide*. Grant, G.A., Ed.; W.H. Freeman and Company: New York, 1992, pp 77-183.

with a tetramethylguanidinium functionality [Guasepohl et al., 1992; Story and Aldrich, 1994; see also chapters 4, 5 and 6].

To obtain optimal efficiency, both phosphonium and uronium reagents require a basic condition and an additive. A tertiary amine, such as *N*-methylmorpholine (NMM) or *N,N*-diisopropylethylamine (DIEA), is commonly used to provide a basic condition [Le-Nguyen et al., 1987; Gausepohl et al., 1988; Fields et al., 1991]. HOBt is an additive reported to further accelerate the rates of BOP and HBTU mediated couplings [Hudson, 1988; Fields et al., 1991]. Recently, 1-hydroxy-7-azabenzotriazole (HOAt, Figure 3.6) has been reported to be an excellent peptide coupling additive [Carpino, 1993]. It enhances coupling yields, reduces racemization and provides visual indication (yellow to colorless) of the reaction endpoint. Several phosphonium and uronium derivatives containing azabenzotriazole were synthesized and reported to be superior to their benzotriazole analogues in solid phase peptide synthesis [Carpino, et al., 1994a,b]. These reagents include HATU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), HAPyU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(tetramethylene)uronium hexafluorophosphate), AOP (7-azabenzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate) and PyAOP (7-azabenzotriazol-1-yl-oxy-tris(pyrrolidino) phosphonium hexafluorophosphate).



HOBt



HOAt

Figure 3.6 Additive reagents used in the formation of peptide bonds

Source: (1) Fields, G.B.; Tian, Z.; Barany, G. Principles and Solid-Phase Peptide Synthesis. In *Synthetic Peptides, A User's Guide*. Grant, G.A., Ed.; W.H. Freeman and Company: New York, 1992, pp 77-183.

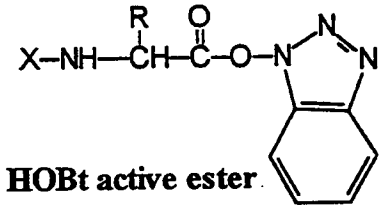
(2) Carpino, L.A.; El-Faham, A.; Minor, C.A.; Albericio, F. Avantageous Applications of Azabenzotriazole (Triazopyridine)-based Coupling Reagents to Solid-phase Peptide Synthesis. *J. Chem. Soc. Chem. Commun.* **1994**, 201-203.

3.3.2 The use of preactivated derivatives

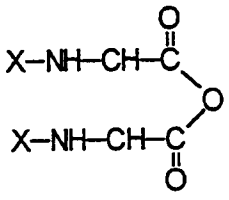
Preactivated *N*-protected amino acids can be prepared in several forms (Figure 3.7). Preformed symmetrical anhydrides, preformed HOBt-active esters and a number of active esters have been evaluated as suitable reagents for solid phase syntheses [Fields et al., 1992]. The lability of most preformed symmetrical anhydrides and of HOBt active esters requires their preparation immediately prior to coupling. In addition, symmetrical anhydrides require two equivalents of protected amino acid to form one equivalent of activated species. Active esters such as nitrophenyl (ONp) esters or pentafluorophenyl (OPfp) esters provide less side reactions. Pentafluorophenyl esters have also been reported to be more reactive than DCC, DCC/HOBt, or symmetrical anhydride coupling [Hudson, 1988].

A new class of preactivated amino acids, the urethane-protected amino acid N-carboxyanhydrides (UNCAs), was introduced by Fuller et al. [1990]. Both Fmoc and Boc-protected N-carboxyanhydrides are stable and crystallaline solids of these derivatives have been use successfully in solid phase synthesis [Fuller et al., 1990; Xue and Naider, 1993]. They are highly reactive toward nucleophiles, and form peptide bonds quickly and cleanly with carbon dioxide as the only byproduct. The rate of Boc-NCA couplings was reported to be comparable to that found for BOP and HBTU mediated couplings [Xue and Naider, 1993].

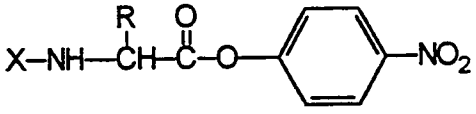
The use of N^{α}-protected amino acid chlorides has been long-known but is limited because the tert-butyl moiety is not completely stable to reagents used for the preparation [Fields et al., 1992]. Thus Boc-amino acid chlorides and Fmoc-amino



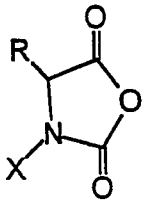
HOBt active ester



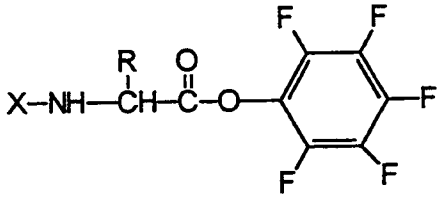
Symmetric anhydride



ONp active ester



Urethane *N*-carboxyanhydride



OPfp active ester

Figure 3.7 Preactivated N-protected amino acids

Source: Fields, G.B.; Tian, Z.; Barany, G. Principles and Solid-Phase Peptide Synthesis. In *Synthetic Peptides, A User's Guide*. Grant, G.A., Ed.; W.H. Freeman and Company: New York, 1992, pp 77-183.

acid chlorides containing side-chain protection with tert-butyl groups are not obtained in stable crystallline forms. Fortunately, Boc and Fmoc groups are stable to acid fluoride preparation with cynauric fluoride [Carpino et al., 1990; Carpino et al. 1991]. Fmoc amino acid chlorides and fluorides react rapidly under solid phase synthetic conditions in the presence of HOBt/DIEA and DIEA, respectively, with very low levels of racemization [Carpino et al., 1990; Carpino and Chao, 1991].

3.4 Final cleavage

Solid phase synthesis using Boc chemistry is designed for simultaneous cleavage of the peptide anchoring linkage and side-chain protecting groups (i.e. benzyl alcohol derivatives) with strong acid. Treatment with liquid hydrogen fluoride (HF) is commonly used in the presence of a carbonium scavenger, usually 10% anisole [Stewart and Young, 1984]. Additional scavengers, such as dimethylsulfide, *p*-cresol, and *p*-thiocresol are used in conjunction with a two-stage "low-high HF" cleavage method that provides extra control and better product purities [Tam et al., 1983]. HF is an extremely toxic acid so this acid should be used only by trained individuals. Therefore many alternative strong acid reagents have been recommended. These strong acid reagents include TFMSA (trifluoromethanesulfonic acid)-thioanisole in TFA [Tam and Merrifield, 1987], TMSBr (trimethylsilyl bromide)-TMSOTf (trimethylsilyl trifluoromethanesulfonate) [Nomizu et al., 1991] and TMSOTf-thioanisole in TFA [Fujii et al., 1987].

For Fmoc/*tert*-butyl syntheses, the *tert*-butyl group and peptide are deprotected and cleaved simultaneously by TFA. It is very common to use cleavage "cocktails" to prevent side reactions which occur because of reattachment of protecting groups to some certain amino acid functionalities, for example alkylation on the indole ring of Trp [King et al., 1990; Choi and Aldrich, 1993]. Two cleavage cocktails typically used are "Reagent K" containing TFA/phenol/thioanisole/ 1,2-ethanedithiol/water (82.5/5/5/2.5/5) [King et al., 1990] and "Reagent R" containing TFA/thioanisole/1,2-ethanedithiol/anisole (90/5/3/2) [Albericio et al., 1990].

3.5 Synthesis of cyclic peptides

There has been considerable interest in the synthesis of cyclic analogues of biologically active peptides because incorporation of such constraints into these peptides may induce a preferred bioactive conformation and may result in improving receptor binding and enhancing biologcal activity [Hruby, 1982; Hruby et al., 1990]. Linear chains of amino acids can form two kinds of cyclic peptides: *homodetic* and *heterodetic* [Kates et al., 1994]. The *homodetic* peptides contain only peptide linkages (lactams) connecting the constituent amino acid residues, whereas the *heterodetic* peptides have other functional groups such as disulfide, ester (lactone), ether, or thioether bridges forming the ring.

3.5.1 Homodetic peptides

Gramicidin S was the first biologically active cyclic peptide which was synthesized chemically [Schwyzer and Sieber, 1957]. Since then a number of

conformationally constrained peptides containing medium- and long-range lactam bridges have been widely prepared. The lactam bridge can involve: (1) side-chain to side-chain cyclization; (2) end-to-end or head-to-tail cyclization; and (3) side-chain to end groups (head or tail) cyclization (Figure 3.8). Using these approaches, the side chain structure or the chirality of amino acids often needs to be modified to prepare a cyclic structure. In many cases it is critical that the side chain group modified not to be one that is essential for biological activity. Thus, an additional topology for cyclization, the "backbone" cyclization (Figure 3.9), has been suggested to overcome these limitations [Gilon et al., 1991].

Homodetic peptides can be prepared either in solution, or the cyclizations performed while the peptides remain attached to the solid supports. The major side reaction found when the linear starting peptide is cyclized in solution is interpeptide condensation, giving dimers and higher oligomers even under high dilution, and in case of head-to-tail cyclization racemization of the C-terminal residue. The cyclization of a peptide on a solid support is more convenient and advantageous because of the *pseudo-dilution* phenomenon that favors intramolecular reaction over intermolecular side reactions [Barany and Merrifield, 1979]. In addition the reagents for cyclization can be used in a large excess and are removed from the product by simple filtration. Several factors, i.e. solid supports, solvents used for the cyclization reaction, coupling reagents, and amino acid sequences, can influence yields and purity of the desired cyclic peptide. Details will be discussed in chapter 4.

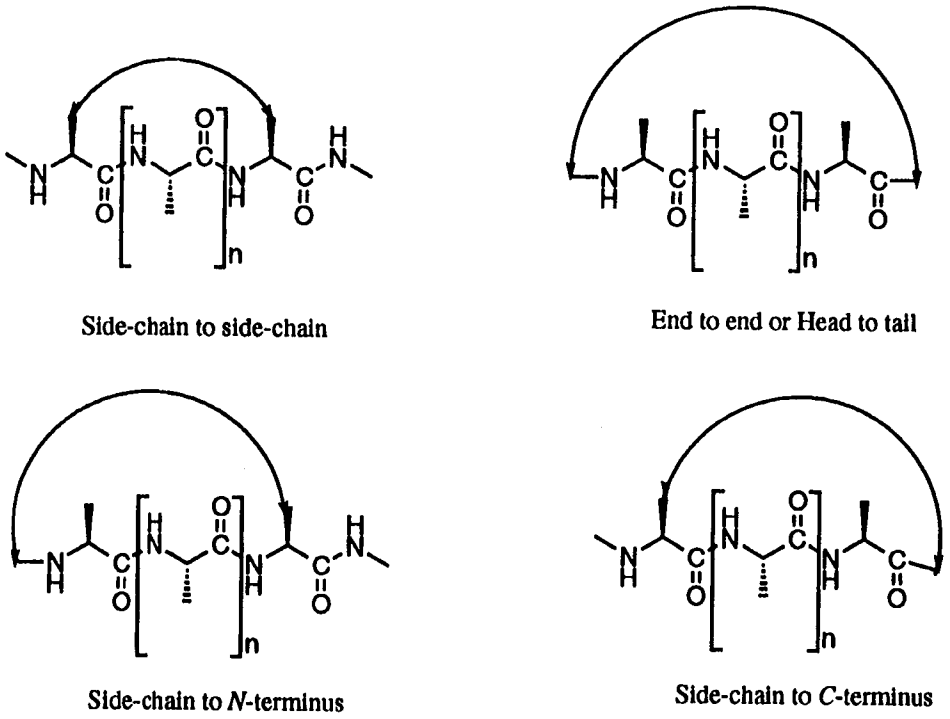


Figure 3.8 The classical modes of *homodetic* peptide cyclization

Source: Gilon, C.; Halle, D.; Chorev, M.; Selinger, Z.; Byk, G. Backbone Cyclization: A New Method for Conferring Conformation Constraint on Peptides. *Biopolymer* **1991**, *31*, 745-750.

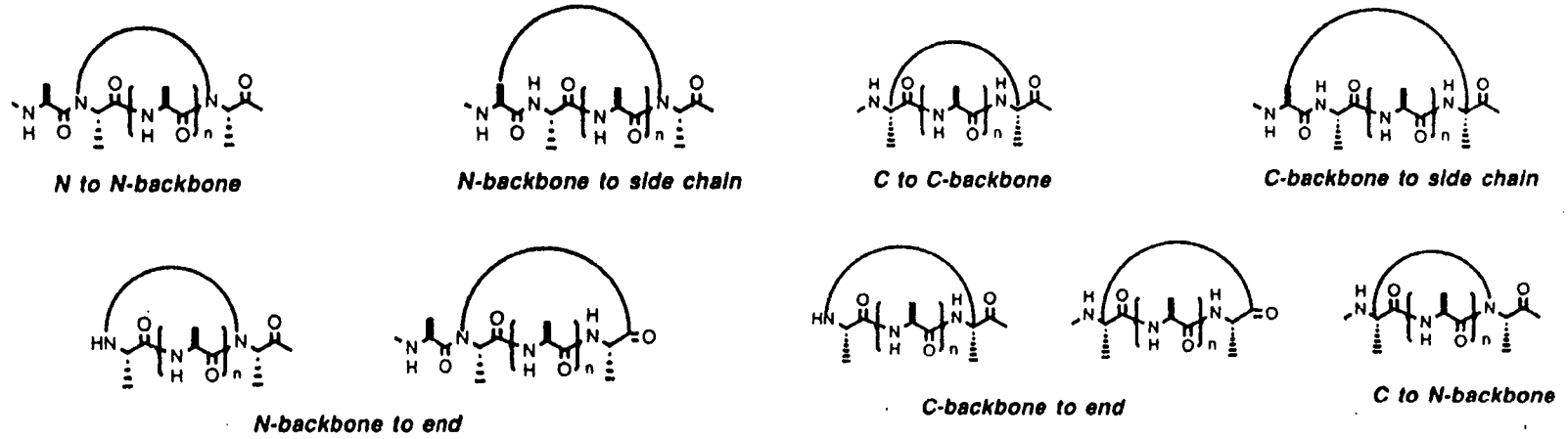


Figure 3.9 "Backbone" cyclizations

Source: Gilon, C.; Halle, D.; Chorev, M.; Selinger, Z.; Byk, G. Backbone Cyclization: A New Method for Conferring Conformation Constraint on Peptides. *Biopolymer* **1991**, *31*, 745-750.

3.6 Evaluation of final product

The identity and purity of the final peptide products have to be evaluated before they can be tested for biological activity. Traditionally, high performance liquid chromatography (HPLC) has been used for checking the purity of peptides, and amino acid analysis, peptide sequencing by Edman degradation and fast atom bombardment mass spectrometry (FAB-MS) have been used for verifying the identity of the desired product [Fields et al., 1992].

During the last 20 years, mass spectrometric methods have been developed for the characterization of peptides and proteins [Biemann, 1986; Biemann, 1988]. Mass spectrometric techniques widely used for peptide and protein characterizations are FAB-MS (fast atom bombardment mass spectrometry), MALDI (matrix assisted laser desorption ionization mass spectrometry) and electrosray mass spectrometry [Biemann and Martin, 1987]. The combination of HPLC on-line with mass spectrometry (LC-MS) makes it possible to evaluate the crude peptide without prior purification [Vestal, 1990]. Tandem mass spectrometry (MS/MS), which combines at least two mass spectrometric instruments, also provides advantages over conventional Edman sequencing in determining the covalent structure of a peptide [Biemann and Scoble, 1987]. Applications of LC-MS and tandem mass spectrometry for evaluating crude peptides obtained from cyclization reactions are described in chapter 5.

CHAPTER 4

SOLID PHASE SIDE-CHAIN TO SIDE-CHAIN CYCLIZATIONS: A COMPARISON STUDY OF ACTIVATING REAGENTS

Seksiri Arttamangkul[1], Leena Leelasvatanakij[1] and Jane V. Aldrich[1,2]

[1] College of Pharmacy
[2] Center for Gene Research and Biotechnology

Abbreviations:

Abbreviations used for amino acids follow the rules of the IUPAC-IUB Joint Commission of Biochemical Nomenclature in *Eur. J. Biochem.* **1984**, *138*, 9-37. Amino acids are in the L-configuration except when indicated otherwise. Additional abbreviations used are as follows: Boc, *t*-butyloxycarbonyl; BOP, benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate; ClZ, 2-chlorobenzyloxy-carbonyl; Dap, α,β-diaminopropionic acid; DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, dichloromethane; DIC, *N,N'*-diisopropylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; DMA, *N,N*-dimethylacetamide; DMF, *N,N*-dimethyl-formamide; Dpg, dipyrrolidinylguanidinium; DPPA, diphenylphosphorylazide; Dyn A, dynorphin A; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, (9-fluorenylmethoxy)carbonyl; HAPyU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(tetramethelene)uronium hexafluorophosphate; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MBHA, 4-methylbenzhydrylamine; PyBOP, benzotriazol-1-yl-oxy-trispyrrolidinophosphonium hexafluorophosphate; PyAOP, 7-azabenzotriazol-1-yl-oxy-trispyrrolidinophosphonium hexafluorophosphate; TBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; Tmg, tetramethylguanidinium; Tos, 4-toluenesulfonyl.

4.1 Abstract

In the synthesis of cyclic peptide analogues, the cyclization reaction is often the yield-limiting step and can produce byproducts which are difficult to separate from the desired peptide. We examined several activating reagents for their efficiency of lactam formation in the synthesis of a cyclic analogue, cyclo[D-Asp^2,Dap^5]Dyn A(1-13)NH_2 (Dap = α,β-diaminopropionic acid), of the opioid peptide dynorphin A (Dyn A). Comparison of the results following cyclization on the resin with BOP (benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate), PyBOP (benzotriazol-1-yl-oxy-trispyrrolidinophosphonium hexafluorophosphate), PyAOP (7-azabenzotriazol-1-yl-oxy-trispyrrolidinophosphonium hexafluorophosphate), HBTU (*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), HATU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate), and HAPyU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(tetramethelene)uronium hexafluorophosphate) indicated significant differences for these activating reagents. Cyclization with HBTU, HATU and HAPyU yielded a major side product, identified as the linear alkyl guanidinium byproducts, [D-Asp^2,Dap(Tmg)^5]Dyn A(1-13)NH_2 for HBTU and HATU, and [D-Asp^2, Dap(Dpg)^5]Dyn A(1-13)NH_2 for HAPyU. A low yield of the desired cyclic peptide was also observed with the use of these uronium reagents. Decreasing the excess of these uronium reagents did not improve the yield of the cyclic peptide. HAPyU gave cleaner peptide products than those obtained from HBTU and HATU, but the Dpg linear byproduct was more difficult to separate from the cyclic peptide than was the Tmg linear byproduct. Comparison of the results for

the cyclizations of different peptides indicated that the more difficult the cyclization, the larger the portion obtained of the linear alkylguanidinium byproduct. In contrast, sufficient cyclic peptide products could be obtained when the phosphonium reagents BOP, PyBOP and PyAOP were used, although these cyclization reactions progressed slowly. All these phophonium reagents yielded similar peptide byproducts which were the unreacted linear peptide and the cyclodimer. PyBOP and PyAOP exhibited faster cyclization reactions than BOP.

4.2 Introduction

Conformational constraint of biologically active peptides via cyclization has been a focus of considerable research and has been extensively employed in the examination of possible bioactive conformations [Hruby et al., 1990]. The cyclic portion may induce a suitable conformation for the entire peptide so that it can adopt the secondary structure required for receptor recognition. Hence, increased receptor selectivity and biological activity are often obtained. In addition, cyclic structures may enhance conformational homogeneity and therefore simplify conformational analysis in solution [Kessler, 1982].

Cyclic peptides can be prepared either by solution or solid phase synthesis. In both methods, the cyclization reaction is generally the yield-limiting step. Cyclizations in solution are commonly limited by dimerization or oligomerization which can occur even at high dilution [Bodanszky and Martinez, 1983]. Cyclizations on a solid support can decrease this side reaction via the "pseudo-dilution" phenomenon [Barany and Merrifield, 1979], so that cyclizations on an insoluble

support will favor intramolecular reactions over intermolecular reactions. More over, in cyclizations on a solid support it is often possible to drive the reactions to completion using excess soluble reagents; soluble reactants and by-products can then be easily removed by filtration without manipulative losses.

There are other factors which can affect the purity and yields of cyclic peptide products. The ring size of the constraint is one of the most important factors. Usually there are no difficulties for cyclizations of peptides containing seven or more amino acid residues [Ehrlich et al., 1993]. For cyclic peptides with smaller ring sizes, the cyclizations are often more difficult, but they can be enhanced by the presence of turn-inducing amino acid residues such as glycine, proline or a D-amino acid [Dale, 1966; Kessler and Haase, 1992]. The amino acid sequence can be another major limiting factor. Schiller et al. [1985b] reported a cyclodimeric peptide as the predominant product in their synthesis of Tyr-cyclo[D-Orn-gly-Glu]-NH_2, even though this cyclization was performed on a solid support and the linear starting peptide contained a D-amino acid. For solid phase cyclization, the loading of the solid support [Plaué, 1990; Ösapay et al., 1990], the extent of cross-linking within the polymeric support and solvents used [McMurray et al., 1994] have all been reported to affect purity and yields of cyclization products. Besides these factors, the formation of a lactam is also dependent upon the efficiency of the activating reagent used. A slow reaction has been reported to result in racemization when the the carboxyl terminus is activated due to prolonged activation [Ehrlich et al., 1993],

while a fast activation may cause dimerization and oligomerization [Plaué, 1990; Schmidt and Neubert, 1991].

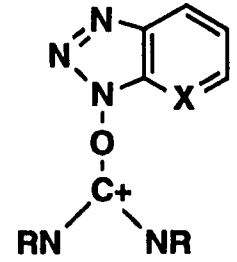
To date a number of activating reagents have been reported for peptide bond formation, but only a few of these have been used for cyclization reactions. DPPA (diphenylphosphorylazide), a modified azide activating reagent, has been used to effectively cyclize peptides, particularly in solution [Al-Obeidi et al., 1989; Schmidt and Neubert, 1991; Hoffmann et al, 1991; Zimmer et al., 1993]. The carbodiimides are well-known reagents for backbone couplings, but their use in cyclization reactions has been limited by slow reaction rates and the frequent production of dimeric products [Schiller et al., 1985; Schiller et al.; 1988; Felix et al., 1988b]. The phosphonium reagent BOP (benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate, see Figure 4.2), introduced by Castro [Castro et al., 1975], is more efficient than the carbodiimides for backbone couplings [Hudson, 1988] and was found to be a more suitable activating reagent than DCC (*N,N'*-dicyclohexyl-carbodiimide) for cyclization reactions due to rapid cyclization rates and the production of the desired cyclic peptides in high yields and purity [Felix et al, 1988b]. In cases of difficult cyclizations, however, the use of BOP was reported to give low yields and low purity of the cyclic peptides [Plaué, 1990; Schmidt and Neubert, 1991].

Several uronium reagents have proved to be excellent coupling reagents for peptide bond formation. In regular backbone couplings, the reaction rates of HBTU (*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, see Figure

4.1) and TBTU (*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) were found to be faster than BOP [Knorr et al., 1989]. Cyclizations using HBTU or TBTU in solution, however, gave less favorable results due to difficulties in purification [Schmidt and Neubert, 1991].

Recently, the introduction of an efficient peptide coupling additive 1-hydroxy-7-azabenzotriazole (HOAt), has resulted in more coupling reagents with high efficency, such as AOP (7-azabenzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate), PyAOP (7-azabenzotriazol-1-yl-oxy-trispyrrolidinophosphonium hexafluorophosphate, Figure 4.1), HATU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, Figure 4.1) and HAPyU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(tetramethelene)uronium hexafluorophosphate, Figure 4.1) [Carpino, 1993; Carpino et al., 1994a]. These reagents show excellent coupling efficiency with low levels of racemization for the synthesis of linear peptides [Carpino et al., 1994a]. The use of HAPyU for cyclizations in solution was found to be superior to DPPA, BOP and TBTU [Ehrlich et al., 1993].

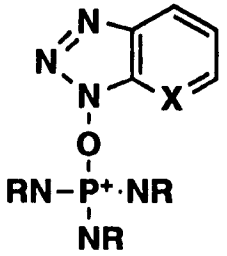
In the present study, we were interested in evaluating different activating reagents, particularly the phosphonium and the uronium derivatives, for the synthesis of lactams on a solid support. We studied the cyclization reaction using the model peptides cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2, **1**, and cyclo[D-Dap^2,Asp^5]Dyn A-(1-13)NH_2, **2**, both of which have 14-membered lactam rings but with the amide linkage in opposite directions, and cyclo[Asp^2,Lys^6]Dyn A-(1-13)NH_2, **3**, which contains a 20-membered lactam ring; structures of the peptides are shown in Figure 4.2. The



HBTU : R = -$(CH_3)_2$, X = CH

HATU : R = -$(CH_3)_2$, X = N

HAPyU : R = -$(CH_2)_4$-, X = N



BOP : R = -$(CH_3)_2$, X = CH

PyBOP : R = -$(CH_2)_4$-, X = CH

PyAOP : R = -$(CH_2)_4$-, X = N

Figure 4.1 Activating reagents used for cyclization study

cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2, **1**

cyclo[D-Dap^2,Asp^5]Dyn A-(1-13)NH_2, **2**

cyclo[Asp^2,Lys^6]Dyn A-(1-13)NH_2, **3**

Figure 4.2 Structures of the cyclic Dyn A model peptides

cyclizations were performed under various conditions using the uronium reagents HBTU, HATU and HAPyU, and the phosphonium reagents BOP, PyBOP and PyAOP (Figure 4.1). The efficiency of these activating reagents, as well as the different byproducts which were formed during the cyclizations, were examined and are discussed in detail below.

4.3 Experimental

4.3.1 Materials

The MBHA (4-methylbenzhydrylamine) resin, BOP and all amino acids except FmocDap(Boc) were purchased from Bachem Inc. (Torrance, CA). FmocDap(Boc) was synthesized in this laboratory as described elsewhere (see chapter 6). *N,N*-Diisopropylethylamine (DIEA) was obtained from Millipore (Bedford, MA). HBTU and PyBOP were purchased from Novabiochem (La Jolla, CA). PyAOP, HATU, HAPyU and HOAt were generous gifts from Dr. Steven Kates, Millipore Corp. DIC (*N,N'*-diisopropylcarbodiimide) and anisole were purchased from Aldrich. Ionate grade TFA was obtained from Pierce (Rockville, IL). All solvents used in peptide synthesis were HPLC grade obtained from Burdick & Jackson (Muskegon, MI).

The peptides were synthesized on a Biosearch 9500 automated peptide synthesizer (Novato, CA), and HF cleavages performed using a Multiple Peptide Systems HF Apparatus model 2010C (San Diego, CA). The peptides were analyzed on a Beckman model 431A gradient HPLC system. The HPLC column was a C_4 Vydac analytical column (300Å, 5μ, 4.6 mm x 25 cm) equipped with a C_4 Vydac guard cartridge. The peptides were eluted using a linear gradient of 10 - 40 %

solvent B in 30 min at a flow rate of 1.0 mL/min and detected at 214 nm; solvent A was 0.1% TFA in H_2O and solvent B was 0.1% TFA in AcCN.

4.3.2 Synthesis of FmocTyr(tBu)-D-Asp(OtBu)-Gly-Phe-Dap(Boc)-Arg(Tos)-Arg(Tos)-Ile-Arg(Tos)-Pro-Lys(ClZ)-Leu-Lys(ClZ)-MBHA

The protected peptide was synthesized on an MBHA resin (1% cross linked polyvinyl styrene, 0.27 mmol/g substitution) using Fmoc amino acids. The synthesis was performed via the standard Fmoc chemistry protocol using DIC/HOBt (4/4 equiv) as the coupling reagent. The following side-chain protecting groups were used: the *t*-butyl ester (OtBu) for D-Asp, Boc for Dap, *t*-butyl ether (tBu) for Tyr, *p*-toluene sulfonyl (Tos) for Arg, and 2-chlorobenzyloxycarbonyl (ClZ) for Lys. The resin (1.0 g) was swollen in DCM for 20 min and then washed with a 10% DIEA in DCM (3 x 30 mL), DCM (5x) and DMA/DCM (1/1, 5x). The repetitive cycle for synthesis was performed by the following steps: (1) addition of the Fmoc-amino acid in DMA (0.4 M) following activation in-line for 2 min with DIC/HOBt (0.4 M of each) in DCM; (2) mixing for 2.0 h (completeness of the reaction was monitored with ninhydrin); (3) washing with DMA/DCM (1/1, 10x); (4) Fmoc deprotection with 30% piperidine in DMA/toluene (1/1; 3 min, then 6 min); and (5) washing with DMA/DCM (1/1, 5x). After the last amino acid, FmocTyr(tBu), was attached, the Fmoc-peptide resin was washed with DCM (5x) and MeOH (5x), and then dried under vacuum prior to further study of the cyclization reactions. An aliquot of this protected peptide-resin was cleaved using HF following Fmoc deprotection (see below) to liberate the linear peptide **5**.

4.3.3 Cyclizations of FmocTyr-D-Asp-Gly-Phe-Dap-Arg(Tos)-Arg(Tos)-Ile-Arg(Tos)-Pro-Lys(ClZ)-Leu-Lys(ClZ)-MBHA, 4

The protected peptide-resin obtained from the synthesis described above (4.3.2) was swollen in DCM for 20 min and the side chain *tert*-butyl protecting groups were cleaved using 50% TFA and 2% anisole in DCM (2 x 25 mL; 5 min and 30 min). The resulting peptide-resin was washed extensively with DCM (5x), MeOH (5x) and DCM (5x), then neutralized using 10% DIEA in DCM (3 x 100 mL) and again washed with DCM (5x), MeOH (5x), and DCM (5x). The peptide-resin was dried under vacuum prior to cyclization.

The dried peptide-resin was divided into portions of 100 mg for the cyclization study. Each portion of the peptide-resin was swollen in DCM/DMF (1/1) for 20 min prior to cyclization. HBTU, HATU or HAPyU were used in either a 4-fold or a 1.5-fold excess relative to the calculated substitution value of the peptide-resin. A 4-fold excess of the HBTU reagent contained additional HOBt (4 equiv) and DIEA (6 equiv) in DMF, whereas a 1.5-fold excess of the HBTU reagent contained HOBt (1.5 equiv) and DIEA (3 equiv) in DMF. Similar compositions were used for both the 4-fold and 1.5-fold excess of HATU and HAPyU reagents, except that HOAt was used in place of HOBt. BOP, PyBOP or PyAOP were used in a 3-fold excess with a 6-fold excess of DIEA, relative to the calculated substitution value of the peptide-resin. The suspension of the peptide-resin and activating reagent was agitated using N_2 for the cyclizations with uronium reagents (HBTU, HATU and HAPyU) or with a mechanical shaker for the cyclizations with phosphonium reagents (BOP, PyBOP and

PyAOP). The activating reagent was replaced with fresh reagent every 24 h when cyclizations were performed for more than one day.

4.3.4 Synthesis and cyclization of FmocTyr(tBu)-D-Dap(Boc)-Gly-Phe-Asp(OtBu)-Arg(Tos)-Arg(Tos)-Ile-Arg(Tos)-Pro-Lys(ClZ)-Leu-Lys(ClZ)-MBHA and FmocTyr(tBu)-Asp(OtBu)-Gly-Phe-Leu-Lys(Boc)-Arg(Tos)-Ile-Arg(Tos)-Pro-Lys(ClZ)-Leu-Lys(ClZ)-MBHA

The protected peptide-resins were synthesized in a similar manner as described in 4.3.2 and 4.3.3. Cyclizations were performed using a 1.5-fold excess of HBTU and HAPyU reagents or a 3-fold excess of BOP reagent.

4.3.5 Peptide cleavage

After the cyclization reaction was terminated, the N-terminal Fmoc group was removed with 30% piperidine in DMA/toluene (1/1; 2 x 10 mL; 5 min and 10 min). The peptide-resin was washed with DMA/DCM (1/1, 10x), DCM (5x) and MeOH (5x) and then dried under vacuum overnight.

The peptide-resin (50 mg) was cleaved using liquid HF (approximately 2 mL) plus anisole (0.2 mL). The HF was allowed to react for 60 min at 0°C. After evaporating the HF at 0°C, the resin was extracted with diethyl ether (3 x 10 mL) and then the cleaved peptide was extracted from the resin with 0.5 M AcOH (3 x 5 mL). The aqueous solution was lyophilized to recover the crude peptide.

4.3.6 Purification and characterization of the peptides

The crude peptides obtained from the cyclization of [D-Asp^2,Dap^5]Dyn A-(1-13)NH_2 using HBTU (4-fold excess) and BOP were desalted by gel filtration on a

Sephadex G-10 column (2.6 x 16.3 cm) in 0.5 M AcOH (monitored at 280 nm), and then lyophilized. These crude peptides were further purified by preparative HPLC (Dupont Zorbax Protein Plus preparative column (C_3, 300 Å, 10 μ, 21 mm x 25 cm) and a Dynamax guard cartridge (C_4, 300 Å, 12 μ, 21 mm x 5 cm)) using a linear gradient of 20-50% solvent B in 30 min at a flow rate of 20 mL/min; solvent A was 0.1% TFA in H_2O and solvent B was 0.1% TFA in MeOH. The pure fractions were collected and lyophilized.

The amino acid compositions of the purified peptides were determined at the Central Services Laboratory, Center for Gene Research and Biotechnology, Oregon State University. All peptides were hydrolyzed using 6N HCl and 1.0% phenol as a scavenger at 110°C for 20 h, then amino acid analyses were performed with post-column ninhydrin detection at 570 nm on a Beckman 126AA System Gold HPLC Amino Acid Analyzer. Edman sequencing was also performed at the Central Services Laboratory using an Applied Biosystems, Model 475A gas-phase protein sequencer system.

Mass spectrometry was utilized to identify all crude peptide products obtained from different cyclizations (see chapter 5). FAB mass spectra (FAB-MS) of the crude and purified peptide products were determined on a Kratos MS-50RF in the positive mode. HPLC-ion spray mass spectrometry was performed on a PE/SCIEX API III Ion Spray triple quadrupole mass spectrometer. The HPLC column was a microbore Vydac C_{18} column and the peptides were eluted using a gradient of 10-100% B in 45 min at a flow rate of 50 μl/min (eluent A was 0.1% TFA in water and eluent B was

0.08% TFA in acetonitrile). For tandem mass spectrometric experiments, samples were introduced into the instrument by flow-injection, with the argon collision gas adjusted to 300 x 10^{13} molecules/cm^2 and an ion energy of 30 eV. Mass spectrometry was performed in the department of Agricultural Chemistry, Oregon State University.

4.4 Results and discussions

Dynorphin A (Dyn A) is a highly potent opioid peptide which is postulated to be an endogenous ligand for κ opioid receptors, but this peptide also binds to other opioid receptor types, i.e. μ and δ receptors [Chavkin, 1982; Goldstein et al., 1979]. Similar to other small linear peptides, Dyn A is a highly flexible molecule and hence can adopt a number of conformations which may contribute to the peptide's low selectivity for κ receptors. According to Schwyzer's hypothesis, Dyn A-(1-13) may adopt a helical structure when bound to the κ receptor type which is postulated to be buried in the lipid-membrane bilayer [Schwyzer, 1986a,b]. Cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2, **1** (Figure 4.1), was therefore designed and synthesized with the hope that the ring portion may induce or stabilize this helical structure.

The synthesis and cyclizations of [D-Asp^2,Dap^5]Dyn A-(1-13)NH_2, **1**, were performed on a solid support in an effort to minimize dimerization and to effect a fast cyclization with a high yield of the desired cyclic peptide. Early attempts to cyclize peptide-resin **4** using BOP reagent (BOP/DIEA; 3/6 equiv) were unsuccessful due to loss of DIEA by evaporation during prolonged nitrogen mixing, but this difficulty was corrected by mechanical shaking in a sealed reaction vessel. The cyclization using BOP reagent was still slow, however, and required 3 days to get nearly complete

reaction (a faint positive ninhydrin and less than 5% of the linear peptide remaining, as determined by reverse phase HPLC following peptide cleavage). We therefore evaluated other activating reagents to determine whether they could increase the cyclization rate and/or yield of the desired cyclic peptide.

4.4.1 Cyclizations using uronium reagents

Since Knorr [Knorr et al.,1989] reported that HBTU is a more efficient coupling reagent than BOP, we examined HBTU reagent (HBTU/HOBt/DIEA; 4/4/6) in an attempt to increase the cyclization rate. A negative ninhydrin was observed within only 2.5 hours for the cyclization reaction using this reagent. Reverse phase HPLC analysis following cleavage showed two major peptide products in approximately a 1:1 ratio (Figure 4.3a). These two peptides were subsequently separated by preparative HPLC and characterized using FAB-MS. The less polar peptide (t_R = 21.3 min) had a molecular ion (M+1) which corresponded with the cyclic peptide **1**, while the molecular ion (M+1) of the more polar peptide (t_R = 18.0 min) was higher than the molecular weight of the cyclic peptide by 116 (Table 4.1). Amino acid analysis, Edman sequencing, HPLC-ion spray mass spectrometry and tandem mass spectrometry (see chapter 5) of the less polar peptide were consistent with that expected for the cyclic peptide (Tables 4.1 and 4.2). Amino acid analysis of the more polar peptide gave correct values for all amino acids except Dap;an earlier peak (t_R 46.3 min) was observed on the chromatogram in place of Dap (t_R 47.8 min) (Table 4.2). This result was similar to that found in our laboratory previously for an enkephalin analogue [Story and Aldrich, 1994] where further

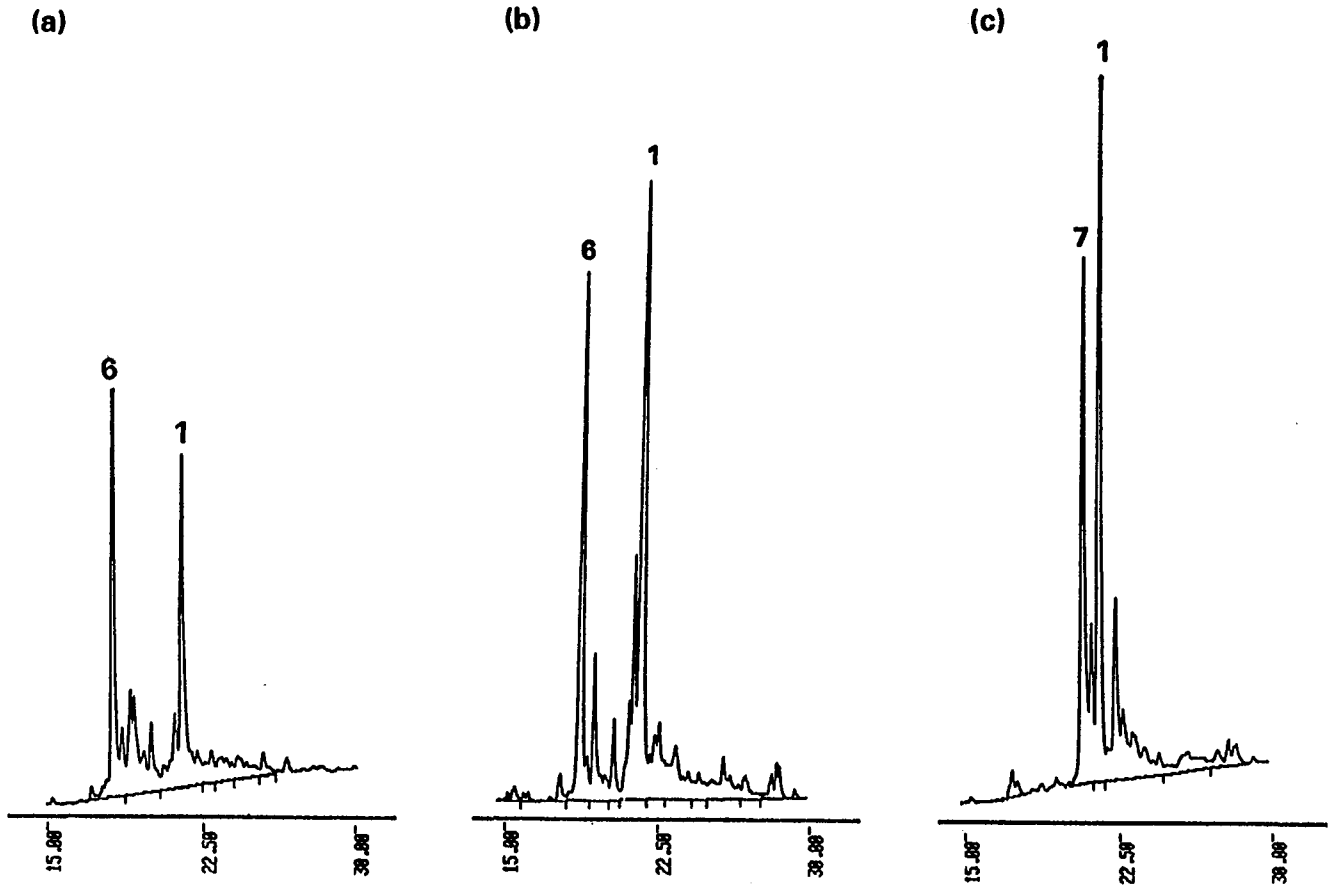


Figure 4.3 HPLC of crude peptides obtained from cyclizations using
(a) HBTU (4-fold excess)
(b) HATU (4-fold excess)
(c) HAPyU (4-fold excess).
Cyclizations were performed for 2.5 - 3 hours.

Table 4.1 Analytical data for the cyclic peptide 1, the Tmg by-product 6, and the linear peptide 5[a]

Peptides	t_R[b] (min)	Mass[c] (M+1)	PTH-amino acids					
			Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
1	21.5	1615.9 (1616)	Tyr	gap	Gly	Phe	d	Arg
6[e]	19.4	1731.9 (1732)	Tyr	Asp	Gly	Phe	f	Arg
5[g]	18.5	1633.8 (1634)	Tyr	Asp	Gly	Phe	Dap[h]	Arg

[a] Theoretical values are shown in parentheses.
[b] From analytical HPLC elution profiles (see Experimental).
[c] Determined from FAB-MS.
[d] t_R = 21.0 min.
[e] The peptide was obtained from HBTU cyclization.
[f] t_R = 28.6 min.
[g] The peptide was obtained from BOP cyclization.
[h] t_R = 23.1 min.

Table 4.2 Amino acid analysis of the cyclic peptide 1, the Tmg by-product 6, and the linear peptide 5

Peptides	Amino acid composition[a]									
	Tyr(1)	Asp(1)	Gly(1)	Phe(1)	Dap(1)	Arg(3)	Ile(1)	Pro(1)	Leu(1)	Lys(2)
1[b]	0.96	1.01	0.99	1.00	0.97	3.03	0.97	0.91	1.02	2.01
6[b]	0.90	0.93	1.00	1.01	c	3.10	1.01	0.90	1.08	2.13
5[d]	0.94	0.96	1.02	1.00	0.95	3.09	0.94	1.29	1.05	2.05

[a] Theoretical values are shown in parentheses.
[b] The peptide was obtained from the HBTU cyclization.
[c] A peak was observed at t_R = 46.3 min; no peak was observed for Dap (t_R = 47.8 min).
[d] The peptide was obtained from the BOP cyclization.

charaterization using high resolution mass spectrometry, ^{1}H-NMR and Edman degradation, indicated that the side chain amino group was converted to the tetramethylguanidinium (Tmg) functional group. Subsequently, we examined the more polar peptide using Edman sequencing (Table 4.1), HPLC-ion spray mass spectrometry and tandem mass spectrometry (see chapter 5) and found that this peptide was the linear tetramethylguanidinium analogue, [D-Asp^2,Dap(Tmg)^5]Dyn A-(1-13)NH_2, **6** (Figures 4.4 and 4.5).

Earlier Gausepohl et al. [1992] reported a tetramethylguanidinium byproduct for a backbone coupling when HBTU reagent was inadvertently added to the amine component. The authors indicated that this reaction resulted from Schiff's base formation between the uronium group and the free amine and could be avoided by using slightly less than one equivalent (e.g. 0.9 equiv) relative to the carboxyl component. We therefore attempted to perform the cyclization of **4** using the HBTU reagents with a lower excess, 1.5 equiv (Figure 4.6) and 0.9 equiv (data not shown). In the former case, the reaction still gave a negative ninhydrin test within 2.5 hours, while a positive ninhydrin was observed after 3 hours in the latter case as expected. In both cases, the Tmg linear peptide **6** was present in higher yields than the cyclic peptide **1**; a 4:1 ratio of Tmg linear peptide **6** to the cyclic peptide **1** was observed for 1.5 equiv of HBTU (Figure 4.6) and a 3:1 ratio for 0.9 equiv of HBTU.

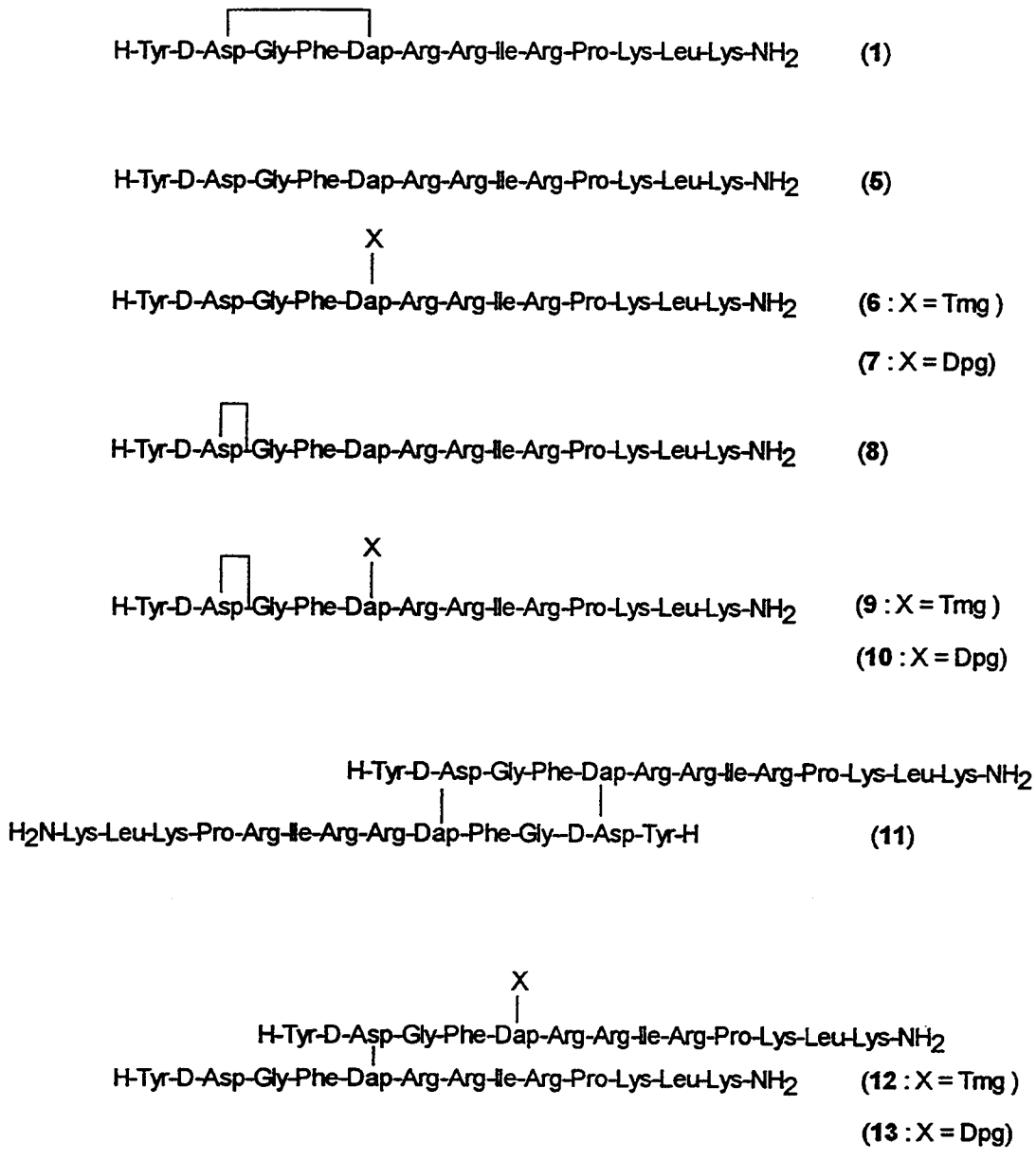


Figure 4.4 Structure of peptide products obtained from cyclizations using different reagents

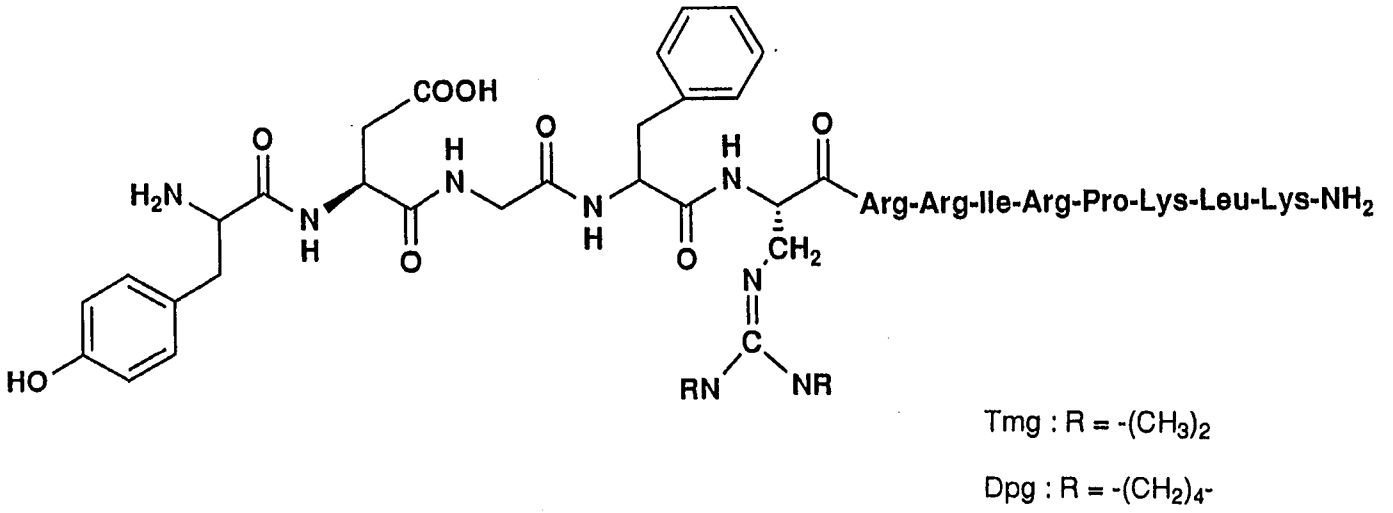


Figure 4.5 Structure of [D-Asp^2,Dap(Tmg)^5]Dyn A-(1-13)NH_2 and [D-Asp^2,Dap(Dpg)^5]Dyn A-(1-13)NH_2

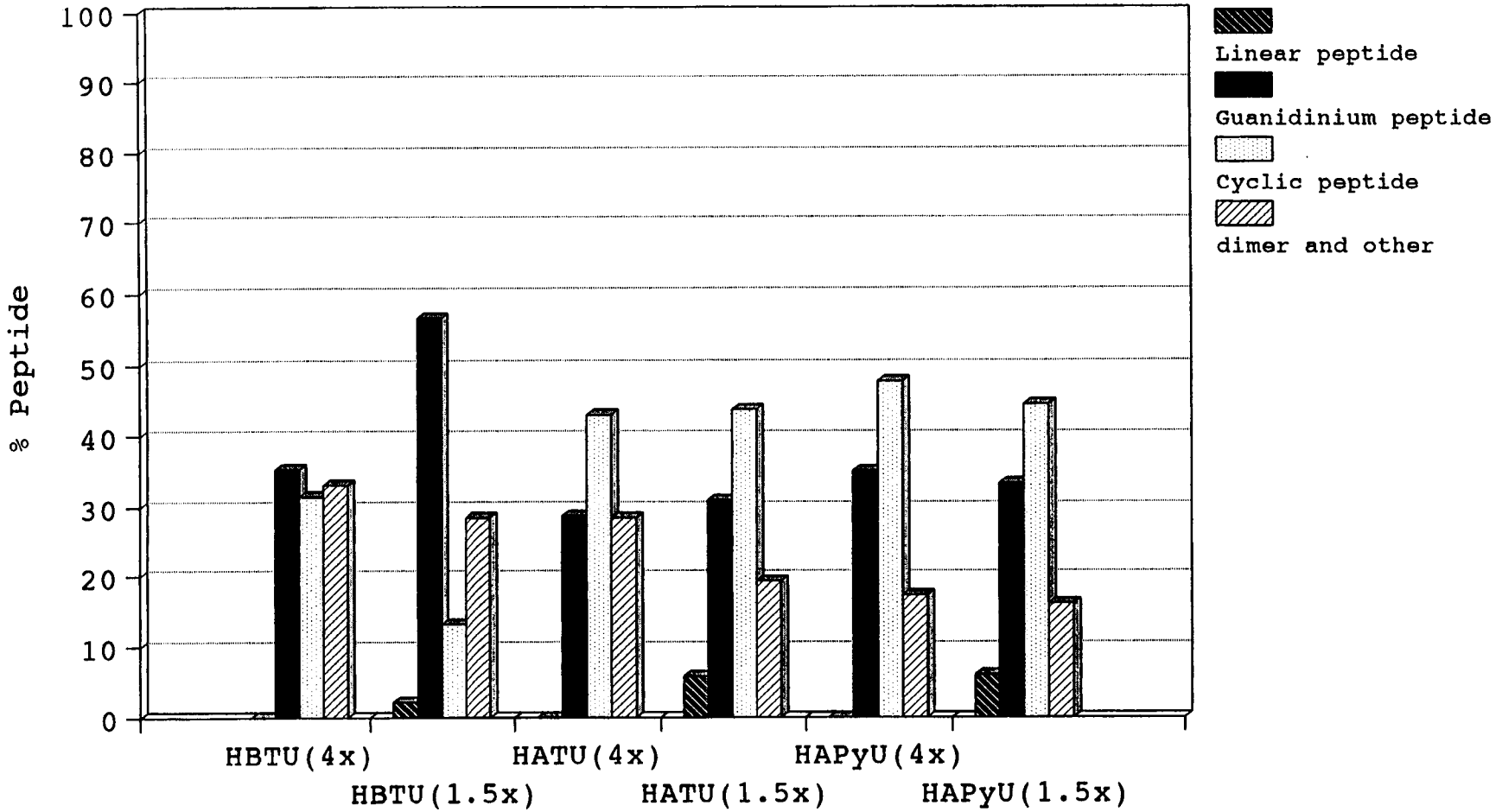


Figure 4.6 Major peptide products from cyclization of [D-Asp^2,Dap^5]Dyn A-(1-13)NH$_2$ using the uronium reagents. Cyclizations were performed for 2.5 hours.

We examined other uronium reagents to determine whether they could be used to perform this particular side-chain to side-chain cyclization on the resin. HATU was chosen to observe the effect of 1-hydroxy-7-azabenzotrizole on cyclization, in comparison with HBTU which contains 1-hydroxybenzotriazole. Carpino et al. [1994a] reported that compared to HBTU, HATU has higher reactivity and is especially suited to the preparation of peptides containing hindered amino acids. A 4-fold excess of HATU reagent (HATU/HOAt/DIEA, 4/4/6) was used to cyclize the peptide-resin **4** and gave a negative ninhydrin reaction within 2.5 hours. An HPLC profile similar to that following HBTU cyclization was observed, except that a slightly higher yield ($\sim 10\%$) of the desired cyclic peptide relative to the Tmg-linear peptide derivative was obtained (Figure 4.3b and Figure 4.6). Similar results were observed when a 1.5 fold-excess of the HATU reagent was utilized, except that the starting linear peptide represented approximately 5% of the peptide products. Therefore, as in the case of HBTU, decreasing the excess of HATU reagent did not decrease formation of the Tmg linear peptide byproduct.

Recently, Enlrich et al. [1993] reported the use of HAPyU for head-to-tail and side-chain to side-chain cyclizations in solution. They found that this activating reagent exhibited superior properties for cyclizations over DPPA, BOP and TBTU. In our study, HAPyU reagent in a 4-fold excess (HAPyU/HOAt/DIEA, 4/4/6) and a 1.5-fold excess (HAPyU/HOAt/DIEA, 1.5/1.5/3) gave a higher yield of the desired cyclic peptide **1** than obtained following HATU cyclizations, but the alkylguanidinium linear peptide was still present as a major contaminant (Figure 4.3c and Figure 4.6).

The structure of this alkylated peptide byproduct was confirmed as the dipyrrolidinylguanidinium (Dpg) peptide [D-Asp^2,Dap(Dpg)^5]Dyn A-(1-13)NH_2, **7** (Figures 4.4 and 4.5), using HPLC-ion spray mass spectrometry and tandem mass spectrometry (see chapter 5). Although HAPyU gave comparable results to HATU, the use of HAPyU for this particular cyclization was limited because the Dpg peptide byproduct was found to be more difficult to separate from the cyclic peptide than was the Tmg peptide byproduct (Figure 4.3c).

These uronium reagents yielded considerable amounts of other byproducts, specifically the cyclodimer **11**, the alkylguanidinium linear dimers **12** and **13**, and the alkylguanidinium linear peptide imides **9** and **10** (Figure 4.4). Since identification of these peptide byproducts was performed using HPLC-ion spray mass spectrometry in which the extent of ionization for each peptide may not be the same (chapter 5), only approximate quantitative information can be obtained. The cyclizations using HBTU and 4 equiv of HATU gave approximately 30% (calculated from HPLC profiles) of these peptide byproducts. When a 1.5-fold excess HATU was used these byproducts decreased by roughly 10% (Figure 4.6). For the cyclizations using HAPyU, only the Dpg linear dimer **13** and the Dpg-linear peptide imide **10** were observed. It was interesting to note that HAPyU also gave lower amounts of these peptide byproducts than did HBTU or 4 equiv of HATU (Figure 4.6).

4.4.2 Cyclizations using phosphonium reagents

As mentioned earlier, BOP reagent (BOP/DIEA, 3/6 equiv) gave a slow cyclization reaction for **4**. Analysis by reverse phase HPLC following cleavage of the

peptide from the resin also revealed that there was another major byproduct besides the linear starting peptide, **5** and the desired cyclic peptide, **1** (Figure 4.7a). The major product at t_R = 21.1 min was purified and characterized using FAB-MS, amino acid analysis and Edman sequencing, which confirmed that this product was the desired cyclic peptide, **1** (Table 4.1 and 4.2). The linear starting peptide, **5**, was identified by comparison of its HPLC retention time (t_R = 17.1 min) with the authentic peptide. HPLC with on-line ion spray mass spectrometry indicated that the peptide byproduct at 20.5 min had a molecular weight corresponding to that of the cyclodimeric peptide **11** (see chapter 5). A time course study using this activating reagent revealed that the cyclization progressed rapidly during the first 0.5 - 1.5 hours and then slowed until that there was only a small increase (< 10 - 15%) in yield of the cyclic peptide after 3 days (Figure 4.8).

Compared to BOP, PyBOP has been reported to be less hazardous and a more effeicient reagent, particularly for coupling hindered amino acid [Coste et al., 1990; Coste et al., 1992]. Therefore, we examined PyBOP reagent (PyBOP/DIEA, 3/6) for cyclization of [D-Asp^2,Dap^5]Dyn A-(1-13)NH_2 on the resin. Analysis by HPLC following cleavage indicated that PyBOP reagent provided similar peptide products, but a slightly faster cyclization reaction, than BOP (Figures 4.7b and 4.9). The crude peptide obtained from cyclization using PyBOP gave more than 60% of the desired

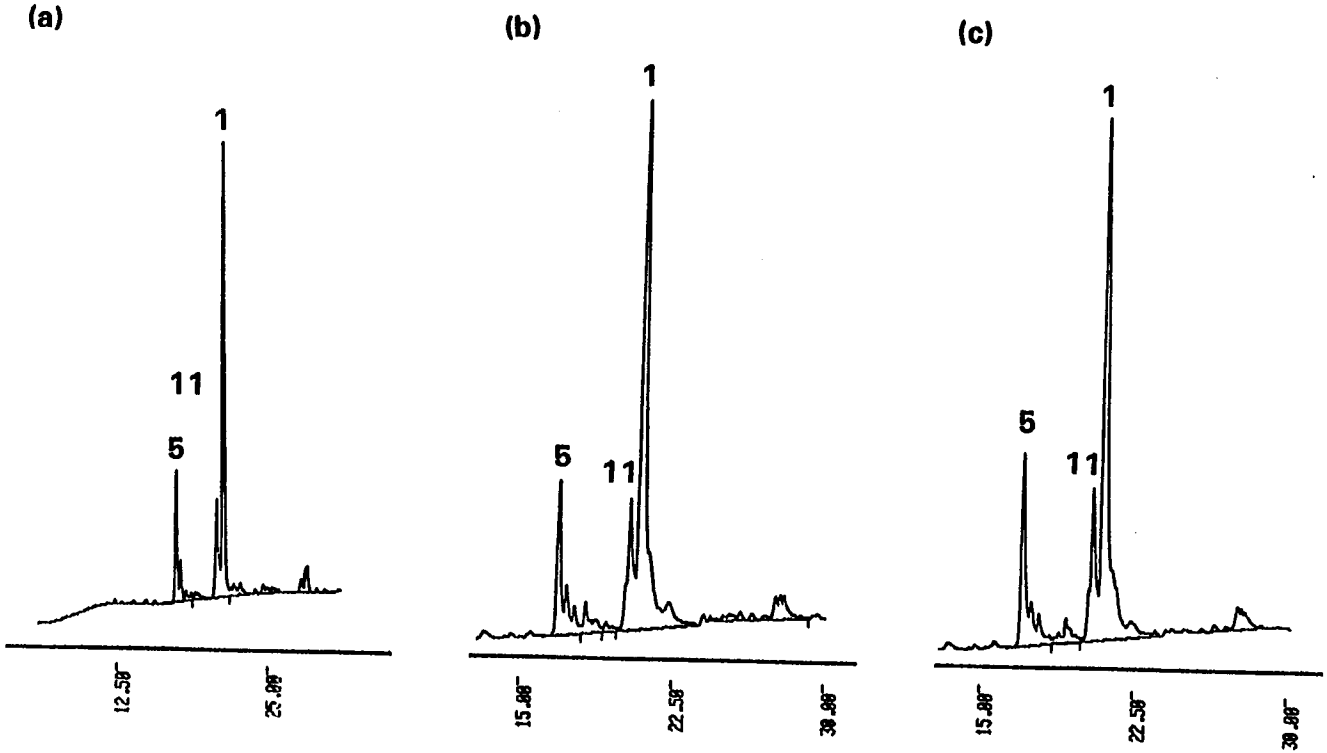


Figure 4.7 HPLC of crude peptides obtained from cyclizations using
(a) BOP (3-fold excess)
(b) PyBOP (3-fold excess)
(c) PyAOP (3-fold excess).
Cyclization were performed for 3 hours.

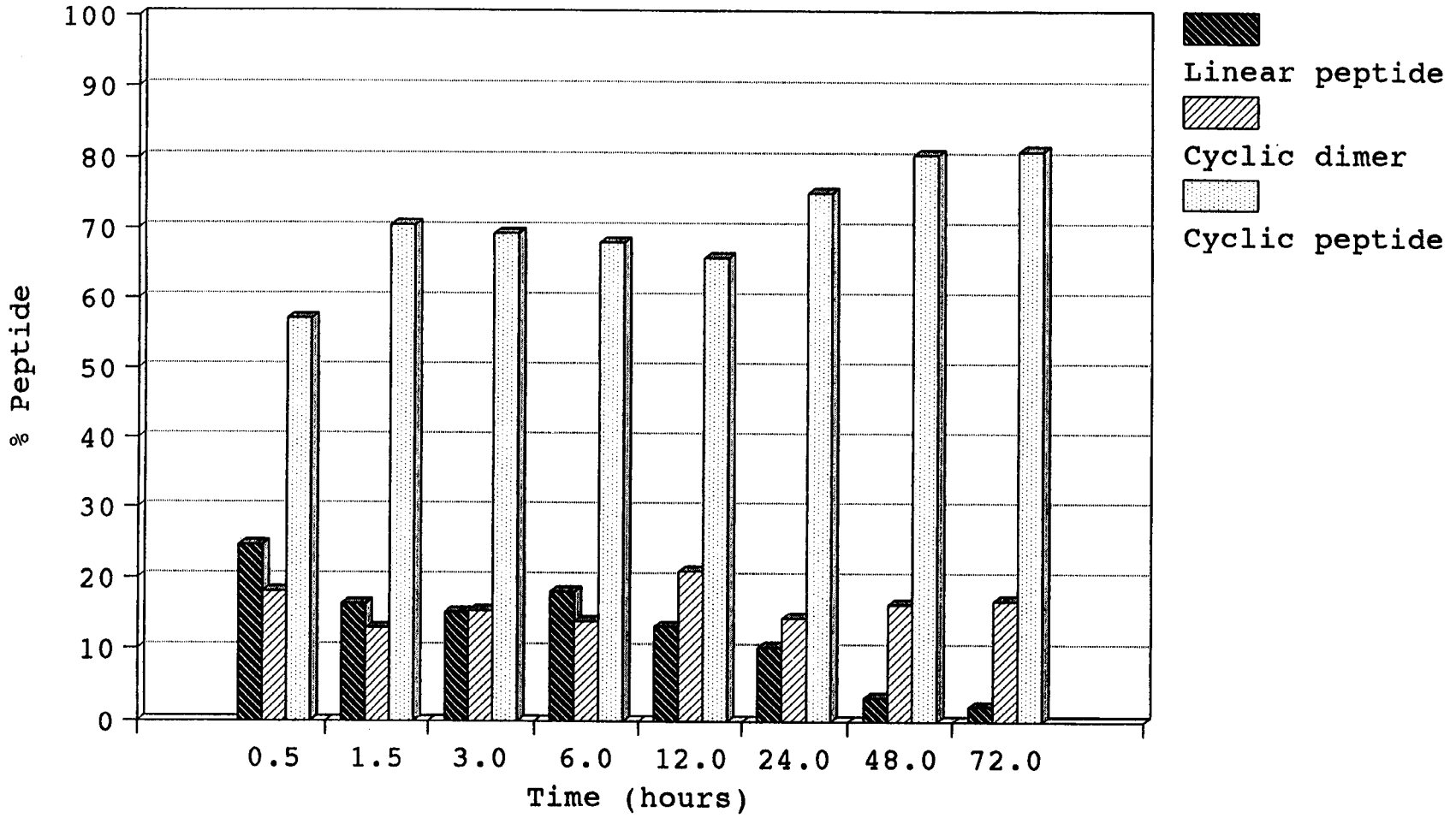


Figure 4.8 Time course of BOP cyclization

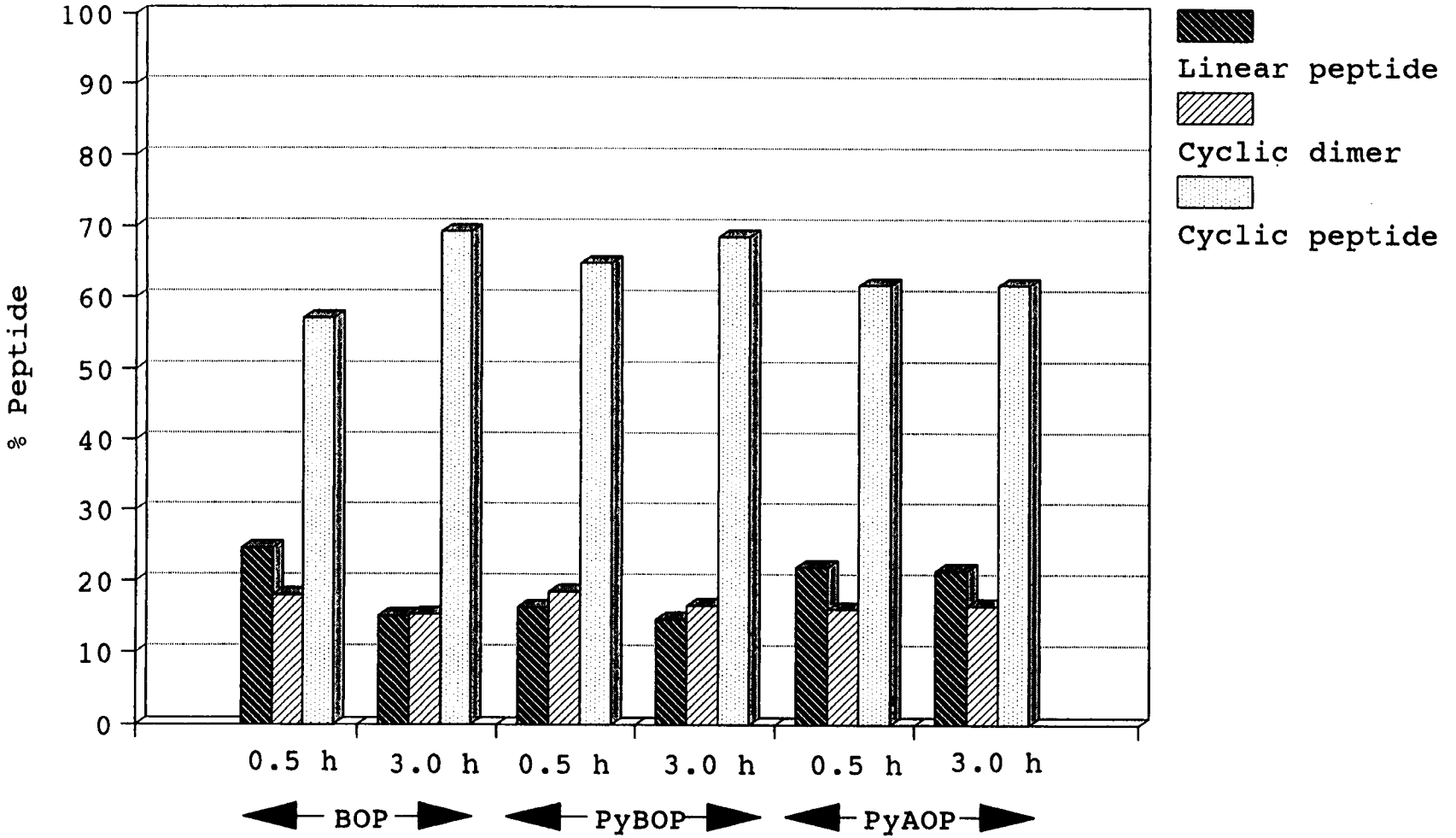


Figure 4.9 Major peptide products from cyclization of [D-Asp^2,Dap^5]Dyn A-(1-13)NH_2 using the phosphonium reagents

cyclic peptide **1** within 0.5 hour, while it required 1.5 hours for BOP reagent to give a similar yield (Figure 4.9).

PyAOP was also evaluated in this study to examine the effect of 1-hydroxy-7-azabenzotriazole in place of 1-hydroxybenzotrizole. A comparable result to that for PyBOP was observed with approximately 60% of the cyclic peptide **1** obtained within 0.5 hour (Figures 4.7c and 4.9). Crude peptides obtained from cyclizations using PyBOP, PyAOP and BOP consistently contained the linear starting peptide **5** and the cyclodimeric peptide **11** in addition to the cyclic peptide **1**. Interestingly, none of these phosphonium reagents affected the yield of the cyclodimeric peptide **11**, which was formed withinin 0.5 hour and remained constant thereafter (Figures 4.8 and 4.9).

Plaué [1990] observed that highly reactive activating reagents tend to promote intermolecular condensation, resulting in dimerized products. The extent of dimerization observed here does not appear to be related to this factor, since similar amounts of cyclodimeric peptide **11** (~20% calculated from the HPLC profile) was also obtained from a cyclization using DIC/HOBt (5 equiv of each, data not shown). The similar results for dimerization from cyclizations using phosphonium reagents, uronium reagents and DIC and the low degree of loading of the MBHA resin used in this study (0.27 mmol/g) suggests that the amino acid sequence and/or conformational constraint of this particular peptide may influence the extent of intermolecular condensation. In addition, some of the peptide chains on the resin must be close together to result in this dimerization. Schiller et al. [1985] indicated that the formation of their dimeric peptide when the cyclization was performed on the resin

may be due to properties of the polystyrene resin which retains some flexibility and could allow two separated peptide chains to come close to each other.

The aspartimide formation observed in this study is not unexpected, since the Asp-Gly sequence is well-known for this rearrangement [Bodanszky and Martinez, 1983; Geiger and Clarke, 1987; Tam et al., 1988; Nicolás et al.,1989]. There are several steps, namely HF cleavage, piperidine deprotection as well as activation of the β-carboxylate on Asp residue, which may determine the extent of this side reaction. The amounts of the aspartimide peptides **8**, **9** and **10** were consistent, accounting for less than 10% of the peptide, suggesting that the activating reagents examined in this study did not affect these aspartimide formations.

4.4.3 Comparison of cyclizations for different peptides

The difficulties observed for the cyclizations described may result from the amino acid sequence and/or the conformational constraint introduced into the linear starting peptide, protected [D-Asp^2,Dap^5]Dyn A-(1-13)-MBHA resin, **4**. To determine the effect of amino acid sequence and ring size, cyclo[D-Dap^2,Asp^5]-, **2**, and cyclo[Asp^2,Lys^6]Dyn A-(1-13)NH_2, **3**, were synthesized. The cyclic peptide **2** contains a 14-membered lactam ring identical to that of cyclic peptide **1** except that the direction of the lactam bridge is reversed, while the cyclic portion of **3** contains a 20-membered lactam ring. Cyclizations were performed in a similar manner to those described for the peptide-resin **4** using BOP reagent (BOP/DIEA, 3/6 equiv), HBTU reagent (HBTU/HOBt/DIEA, 1.5/1.5/3 equiv) or HAPyU reagent (HAPyU/HOAt/DIEA, 1.5/1.5/3 equiv) and a reaction time of 2.5 - 3 hours.

Cyclizations for the reverse-position lactam were found to be even more hindered and gave very low yields of the desired cyclic peptide **2** (Figure 4.10). Analysis using HPLC-ion spray mass spectrometry showed only 20% of cyclic peptide obtained from cyclization using BOP reagent. This crude peptide contained almost 20% of the remaining linear starting peptide plus unidentified side products. Cyclizations using a 1.5-fold excess of HBTU and HAPyU gave the Tmg-linear peptide, [D-Dap(Tmg)^2,Asp^5]Dyn A-(1-13)NH_2 and the Dpg-linear peptide, [D-Dap(Dpg)^2,Asp^5]Dyn A-(1-13)NH_2, respectively, as the major peptide products ($\sim$40% of each, calculated from the HPLC profiles) and only 10% of the desired cyclic peptide **2**. Similar to the cyclizations to give **1**, the cyclization to give **2** using HBTU and HAPyU still yielded considerable amounts of the alkylguanidinium linear peptide imides and the dimeric peptide byproducts.

We further examined these three activating reagents for cyclization of the Dyn A analogue [Asp^2,Lys^6]Dyn A-(1-13)NH_2, **3**, which has a larger ring size. Cyclization using BOP gave a negative ninhydrin test within 3 hours, indicating that this cyclization was more rapid than that observed for the other two peptides. HPLC analysis of the crude peptide revealed only 5% of remaining linear peptide and 55% of the desired cyclic peptide (Figure 4.10). The cyclic peptide obtained from this cyclization was separated and identified using FAB-MS and amino acid analysis. Although the desired cyclic peptide **3** was obtained as the major component, cyclizations using a 1.5-fold excess of HBTU or HAPyU still gave the Tmg- or Dpg-linear byproducts in approximately 15 -18% yield (Figure 4.10). The dimeric peptide

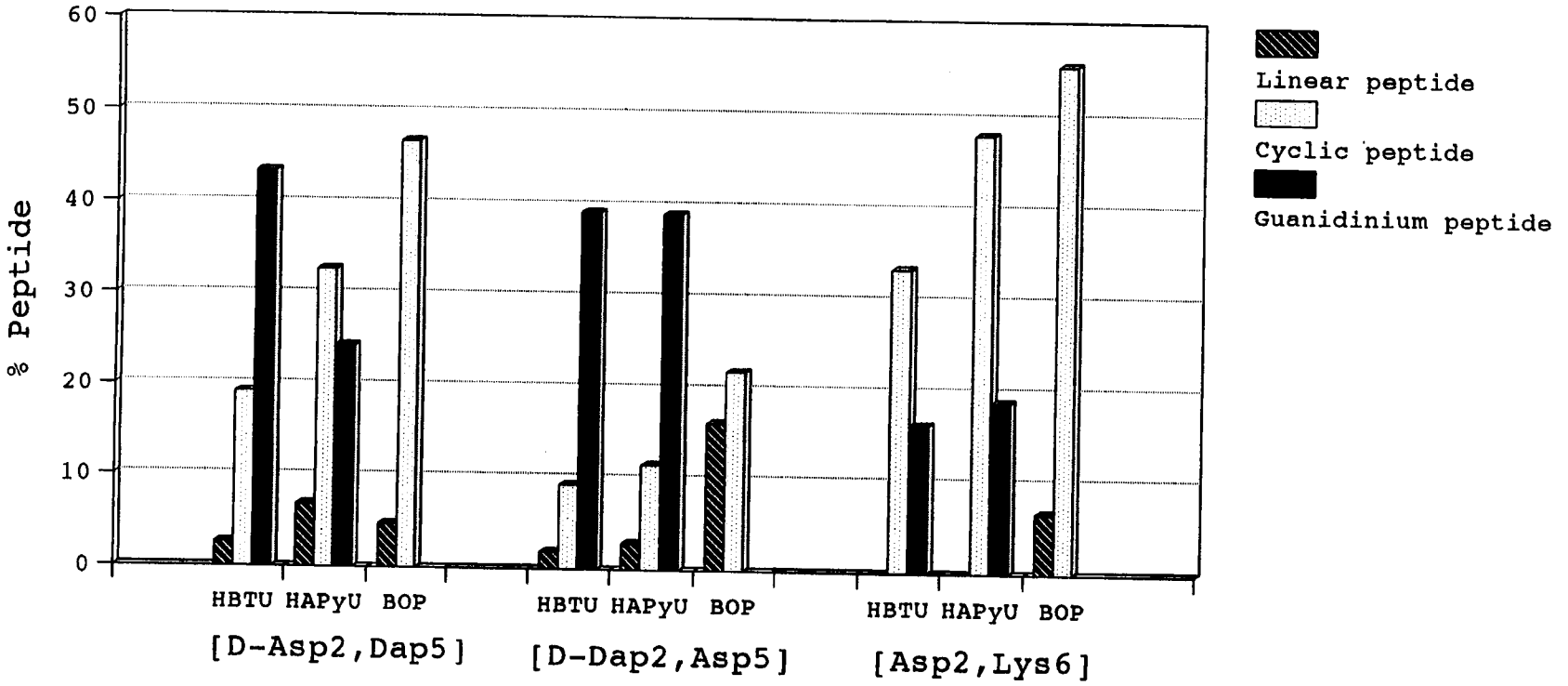


Figure 4.10 Comparison of cyclizations of cyclic peptides with different cyclic constraints. The activating reagents were used as following

(1) HBTU (1.5-fold excess)
(2) HAPyU (1.5-fold excess)
(3) BOP (3-fold excess)
All cyclizations were performed for 2.5 - 3 hours.

products (~15%) and the alkylguanidinium linear peptide imides (~5 - 10%) were also observed following cyclization with these reagents.

4.5 Conclusions

The phosphonium and uronium reagents examined in this study exhibited marked differences in efficiency and applicability for use as activating reagents for lactam formation. Cyclizations to give **1** using the uronium reagents HBTU, HATU and HAPyU gave negative reponses to ninhydrin within only 2.5 hours, but the yield of the desired cyclic peptide was low due to the production of major linear peptide byproducts which contain an alkylguanidinium group (Tmg from HBTU and HATU, and Dpg from HAPyU). Of the three uronium reagents, HBTU gave the poorest results, whereas HAPyU and HATU gave higher yields of the desired cyclic peptides. HAPyU gave a cleaner reaction than HATU, but the Dpg linear byproduct **7** was more difficult to separate from the cyclic peptide **1** than was the Tmg linear byproduct **6**. Decreasing the excess of these uronium reagents did not improve the yield of the desired cyclic peptide.

In contrast to the uronium reagents, the cyclizations to give **1** using the phosphonium reagents BOP, PyBOP and PyAOP were slow reactions which required 3 days to get nearly complete reaction. The cyclizations using PyBOP and PyAOP were slightly faster than that using BOP. At longer reaction times all of these phosphonium reagents gave similar amounts of the peptide products, the desired cyclic peptide, the remaining linear starting peptide, the linear peptide imide and the cyclodimer.

Comparison of the results for the cyclizations of different peptides indicated that the more difficult the cyclization, the larger the portion of the guanidinium byproducts which formed when the uronium reagents were used for the cyclization. This result suggested that in cyclizations using the uronium reagents, longer exposure of free amine to these reagents could promote this side reaction. The formation of these unusual byproducts decreased the yields of the desired cyclic peptides compared to cyclizations with the phosphonium reagents. The formation of guanidinium byproducts limits the usefulness of the uronium reagents for cyclization, particularly in cases where the cyclization is difficult. Although the cyclizations using the phosphonium reagents can be slower, these reagents are more suitable and give greater yields of the desired cyclic products.

CHAPTER 5

CHARACTERIZATION OF SYNTHETIC PEPTIDE BYPRODUCTS FROM CYCLIZATION REACTIONS USING ON-LINE HPLC-ION SPRAY MASS SPECTROMETRY AND TANDEM MASS SPECTROMETRY

Seksiri Arttamangkul[1], Brian Arbogast[2], Douglas Barofsky[2], Jane V. Aldrich[1,3]

[1] College of Pharmacy
[2] Agricultural Chemistry, College of Science
[3] Center for Gene Research and Biotechnology

Abbreviations:

The nomenclature used for peptide fragment ions (positive ions) is described by K. Biemann in *Meth. Enzym.* **1990**, *193*, 886-887 (see Appendix A). Abbreviations used for amino acids follow the rules of the IUPAC-IUB Joint Commission of Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9-37). Amino acids are the L-configuration except when indicated otherwise. Additional abbreviations used are as follows: BOP, benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate; Dap, α,β-diaminopropionic acid; DIEA, *N,N*-diisopropylethylamine; Dpg, dipyrrolidinylguanidinium; Dyn A, dynorphin A; FAB-MS, fast atom bombardment mass spectrometry; HAPyU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(tetramethylene)uronium hexafluorophosphate; HATU, *O*-(7-azabenzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; MBHA, 4-methylbenzhydrylamine; MS-MS, tandem mass spectrometry; Tmg, tetramethylguanidinium.

5.1 Abstract

The cyclization reaction to give a cyclic dynorphin A analogue (cyclo[D-Asp^2,Dap^5]Dyn A(1-13)NH_2) proved to be difficult and yielded substantial byproducts which varied depending upon the activating reagent used. On-line HPLC-ion spray mass spectrometry was found to be more practical and useful than conventional HPLC alone for characterizing the products of these cyclization reactions. Several peptide byproducts were identified from the series of multiply charged ions observed even when some of these peptides eluted at the same retention time. The peptide byproducts observed from cyclizations using BOP (benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate), HATU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and HAPyU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(tetramethylene)uronium hexafluorophosphate) were dimeric peptides, peptides involving aspartimide rearrangement, and tetramethylguanidinium (Tmg) and dipyrrolidinylguanidinium (Dpg) derivatives resulting from alkylation of the side chain of Dap by HATU and HAPyU, respectively. Peptide sequencing by ion spray tandem mass spectrometry was performed to comfirm the structure of some peptide byproducts in the crude sample. A unique fragmentation for the β,γ-bond of the Dap side chain was demonstrated and could be used to identify linear peptide byproducts. In addition, distinctive fragment ions from this cleavage were observed for the Tmg and Dpg functionalities.

5.2 Introduction

The determination of a peptide's identity and purity following peptide synthesis is a prerequisite prior to evaluating a peptide's biological activity and/or physical properties. Standard methods commonly utilized for establishing a peptide's homogeneity and identity are high performance liquid chromatography (HPLC), fast atom bombardment mass spectrometry (FAB-MS), amino acid analysis and, when necessary, peptide sequencing by Edman degradation. Recently capillary electrophoresis (CE) has been introduced for evaluating peptide purity. Because it separates peptides by an orthogonal mechanism to reverse phase HPLC, it is a complimentary technique which can be useful for separating peptides with similar hydrophobicities [Colburn et al., 1991].

When complex mixtures are obtained following peptide synthesis, isolation and identification of individual products can be laborious and time consuming, and can be extremely difficult for products with similar retention times on HPLC. Liquid chromatography combined with on-line mass spectrometry (LC-MS) and tandem mass spectrometry (MS-MS) are alternative methods to conventional HPLC analysis with UV detection and Edman sequencing, respectively, for peptide characterization. These methods have been used successfully to characterize native [Kim et al., 1984; Krishnamurthy et al., 1989] and chemically modified proteins [Biemann and Scoble, 1987], synthetic peptides and their byproducts, and peptide libraries [Metzger et al., 1993; Beck-Sickinger et al., 1991; Papayannopoulos and Biemann, 1992]. Unlike conventional HPLC, HPLC-MS is capable of detecting the presence of two or more

peptides eluting at the same retention time. For a complex mixture, peptide characterization using HPLC-MS has the limitation that peptides with identical molecular weights (isobaric peptides) cannot be distinguished. Tandem mass spectrometry, the technique that provides sequence information, is more useful to confirm and verify peptide structure [Biemann and Scoble, 1987]. It has advantages over Edman sequencing in cases where the N-terminus of the peptide is blocked or when unusual amino acid modifications are involved. Using this powerful technique, the isobaric amino acids Lys and Gln, Leu and Ile, and even α- and β- aspartyl, and α- and γ-glutamyl peptides can be differentiated [Johnson et al., 1987; Lloyd et al., 1988].

In our study of the cyclization of an analogue of the opioid peptide dynorphin A (Dyn A) to give cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2 (Dap = α,β-diaminopropionic acid), different byproducts were obtained depending upon the activating reagent used. Isolation of every peptide byproduct from each synthesis for conventional characterization was impratical. Therefore we utilized HPLC combined with on-line ion spray or "nebulization-assisted" electrospray mass spectrometry to rapidly identify the different side products obtained following the cyclization reactions using different activating reagents. Since ion spray mass spectrometry provides only molecular masses of these peptide byproducts, ion spray tandem mass spectrometry was therefore performed to establish the structures of some unusual peptide byproducts. Evidence from mass spectrometric determinations indicated that depending upon the reagent used dimeric peptides, byproducts involving an

aspartimide rearrangement and/or derivatives resulting from alkylation of the free amine on the Dap side-chain were formed. These results are discussed in detail below.

5.3 Experimental

5.3.1 Peptide synthesis

All of the peptides were synthesized using solid phase synthesis as described in detail in chapter 4. Fmoc-amino acids were assembled on an MBHA (4-methylbenzhydrylamine) resin by standard Fmoc protocol using DIC/HOBt (4/4 equiv, DIC = *N,N*-diisopropylcarbodiimide and HOBt = 1-hydroxybenzotriazole) for backbone couplings. After removal of t-butyl and Boc side-chain protecting groups, the lactam bridge was formed using either BOP (benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate) [Castro et al., 1975], HATU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) or HAPyU (*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(tetramethelene)uronium hexafluorophosphate) [Carpino, 1993] as the activating reagents. The BOP reagent contained BOP/ DIEA (3/6 equiv, DIEA = *N,N*-diisopropylethylamine), the HATU reagent contained HATU/HOAt/DIEA (4/4/6 equiv, HOAt = 1-hydroxy-7-azabenzotriazole), and the HAPyU reagent contained HAPyU/HOAt/DIEA (4/4/6 equiv). The reaction appeared to be complete after 2.5 h for HATU and HAPyU and was terminated after 12 h for BOP. After HF cleavage, crude peptides were extracted and lyophilized. The linear

and cyclic peptide were purified using HPLC as described in chapter 4. Other peptides were used in crude form in further experiments.

5.3.2 Mass spectrometry

FAB mass spectra were obtained on a Kratos MS-50RF mass spectrometer operating at a mass resolution of 1700. The Ion-Tech ion gun was operated at 7-8 keV, using xenon to generate the primary ionizing beam. 3-Nitrobenzyl alcohol with or without 2% trifluoroacetic acid, or 2:1 thioglycerol:glycerol were used as FAB matrices. Usually a small amount of the solid material was placed directly in the matrix on the stainless steel probe tip. LC-MS was performed on a PE/SCIEX API III Ion Spray triple quadrupole mass spectrometer; ion spray voltage was 5000 eV. The HPLC column was a 1 mm Vydac C_{18} column with 5 μm particle size and 300 Å pore size. All peptides were eluted within 20 min using a gradient of 10-100% B in 45 min at a flow rate of 50 μl/min (eluent A was 0.1% TFA in water and eluent B was 0.08% TFA in acetonitrile). The orifice potential was 80-100 eV. The multiply charged ions observed were transformed using automated Hyper Mass, a deconvolution software from PE/SCIEX, to a singly charged ion. For tandem mass spectrometric experiments, samples were introduced into the instrument by flow-injection; the argon collision gas thickness was adjusted to 300 x 10^{13} molecules/cm^2 and the ion energy was 30 eV. Precursor ions selected were doubly charged at *m/z* 808 (purified cyclic peptide, **2**), *m/z* 818 (purified linear peptide, **1**), *m/z* 858 and 867 (crude peptide obtained from HATU cyclization), and *m/z* 893 (crude peptide obtained from HAPyU cyclization).

5.4 Results and discussion

In the study of the cyclization reaction of cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2, we observed different peptide products when different activating reagents (BOP, HATU or HAPyU) were used (Figure 5.1). The reverse phase HPLC chromatograms for the crude peptides were complex (Figure 5.2) and separation of the different products proved to be difficult. The linear peptide, **1** (t_R = 18.8 min) and cyclic peptide, **2** (t_R = 22.5 min) obtained from the BOP cyclization were successfully isolated and their structures verified by FAB-MS, amino acid analysis and Edman sequencing (see chapter 4). When the cyclization was performed using HBTU (*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) as the activating reagent, a linear byproduct **4** resulting from transfer of the uronium group from HBTU to the Dap side-chain was isolated and characterized [Arttamangkul et al., submitted (chapter 6)]. Purification of other peptide byproducts present as shoulders on major peaks or as minor peaks in the HPLC chromatograms was impractical. FAB-MS of the crude peptide mixtures from three different cyclization reactions (BOP, HATU and HAPyU) gave one major peak with an *m/z* of 1615.8, corresponding to the expected mass of the cyclic peptide **1**. Higher mass molecular ions were found as minor peaks (intensity < 10% compared to the peak at 1615.8) at 1731.9 and 1784.0 in the crude peptide mixtures obtained from cyclizations with HATU and HAPyU, respectively. The molecular ion at 1731.9 from the HATU cyclization was consistent with the linear Tmg peptide by-product **4**, whereas the peak

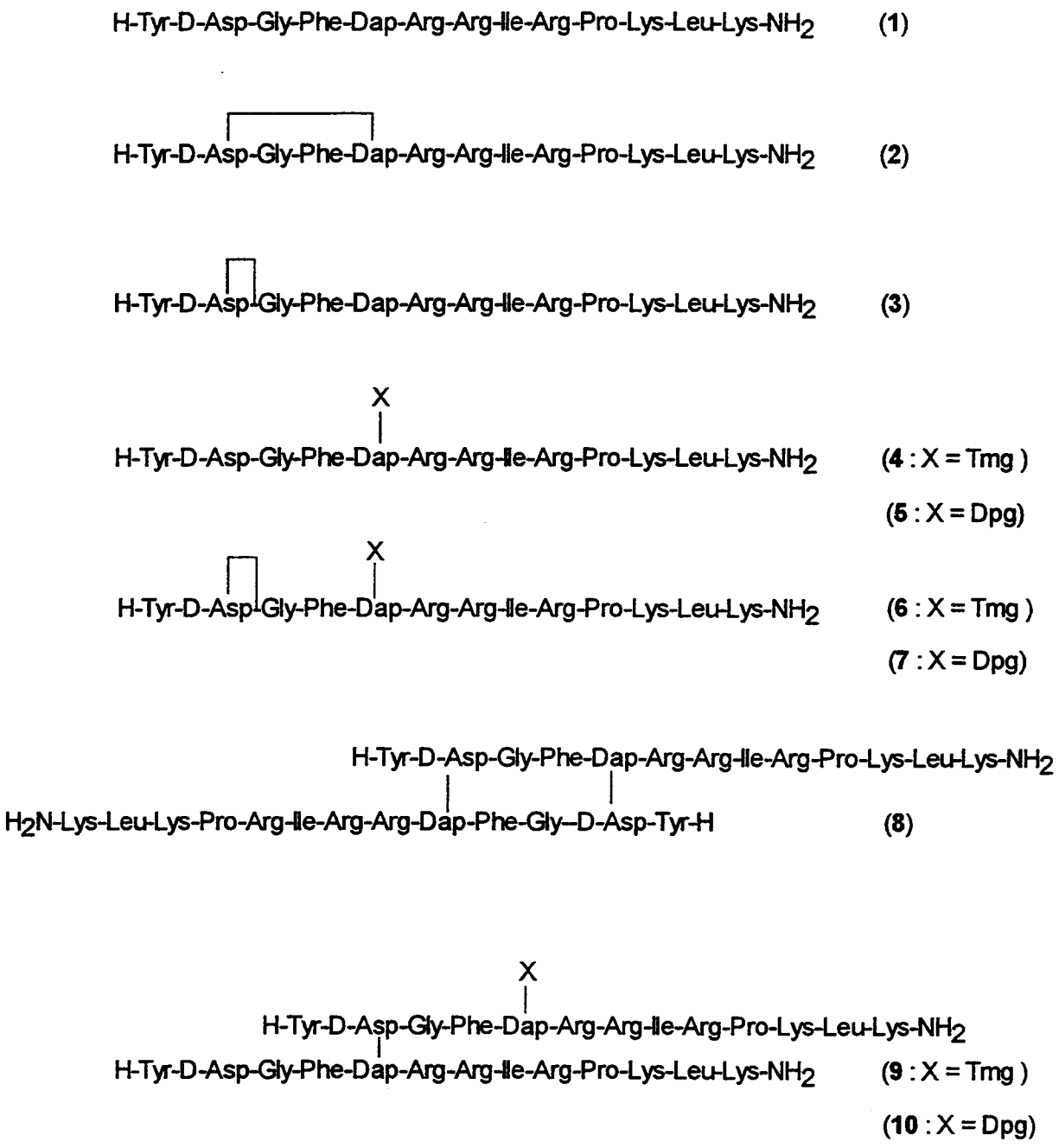


Figure 5.1 Peptide products obtained from cyclizations using BOP, HATU, and HAPyU

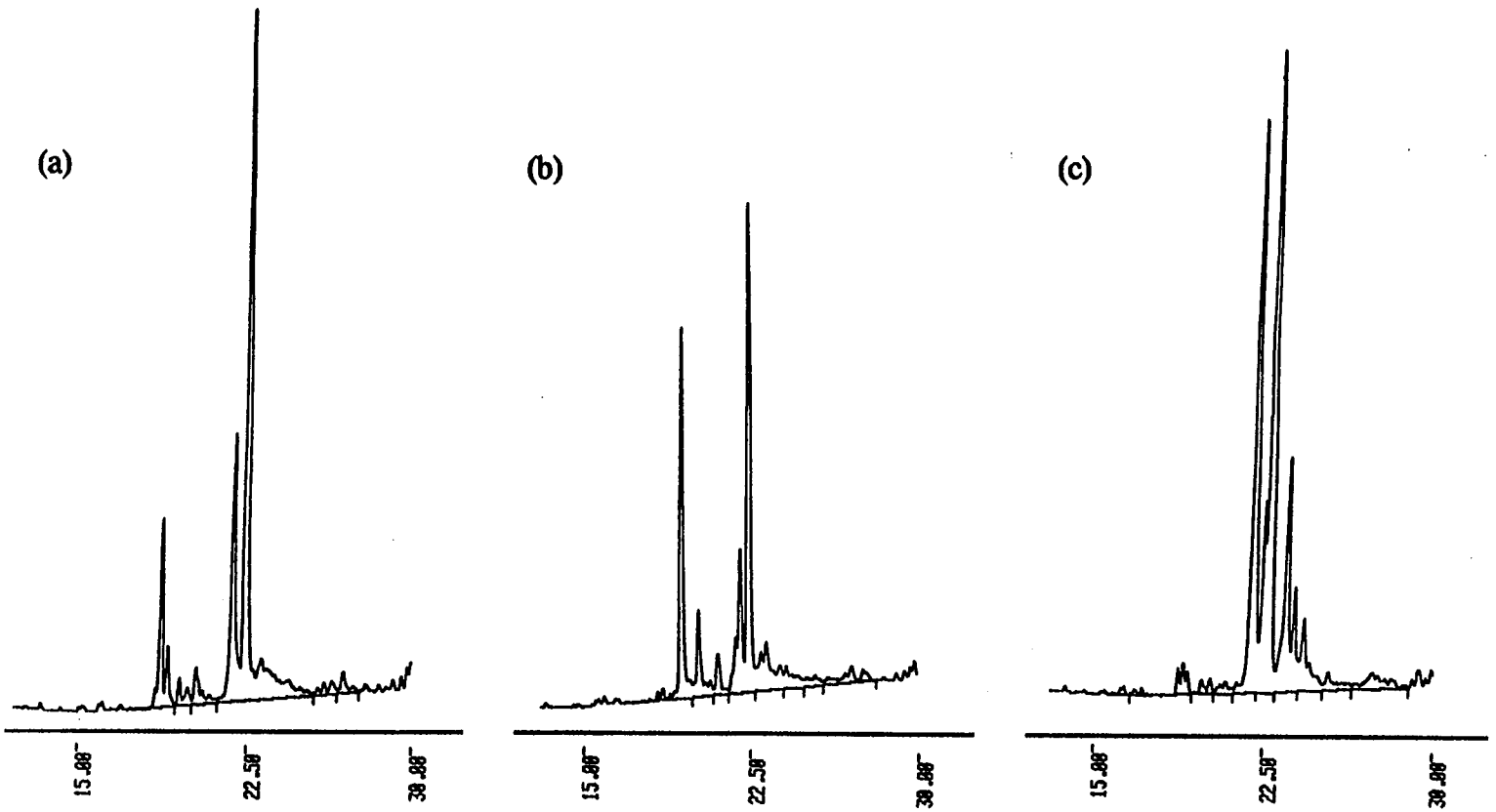


Figure 5.2 HPLC profile of crude samples obtained following cyclizations using different activating reagents. The column was a Vydac 218TP54 (C_{18}) column eluted with a gradient of 10-40% acetonitrile with 0.1%TFA over 30 min at a flow rate of 1.0 mL/min: products following cyclization with (a) BOP; (b) HATU; and (c) HAPyU.

at 1784.0 from the HAPyU cyclization was consistent with the related alkylated peptide by-product **5** with a dipyrrolidinylguanidinium (Dpg) functionality.

Reverse phase HPLC combined with on-line ion spray mass spectrometry was utilized to determine molecular masses of the unresolved peptide byproducts. Total ion counts (TIC) of all masses between 300 to 2000 were collected and are shown in Figure 5.3. Multiply charged ions were deconvoluted to obtain the mass of the parent compounds [Mann et al., 1989]; the results obtained from HPLC-MS following cyclizations using BOP, HATU and HAPyU are summarized in Tables 5.1 - 5.3.

HPLC-MS of the crude peptide obtained from cyclization of [D-Asp^2,Dap^5]Dyn A-(1-13)NH_2 using BOP reagent indicated diverse peptides in addition to the desired cyclic peptide **2** (Figure 5.3a and Table 5.1). Since the cyclization reaction with BOP was not complete after 12 hour, the linear peptide [D-Asp^2,Dap^5]Dyn A-(1-13)NH_2, **1** (t_R = 9.3 min) was detected in the deconvoluted spectrum. A peak with a calculated mass of 1614.8 with a relatively low intensity (< 5% of TIC) eluted shortly after the linear peptide at 9.9 min. This mass is identical to that of the cyclic peptide, **2** (t_R = 11.1 min) but the different retention times indicate that these are different peptide products. Since the mass corresponds to the molecular weight of the linear peptide minus 18, this suggests the presence of the aspartimide peptide **3**. The major component of the peak eluting at 10.7 min appeared to be the cyclodimeric peptide, **8**, which could be distinguished from the cyclic monomer peptide **1** (t_R = 11.1 min) based on the ions carrying an odd number of charges.

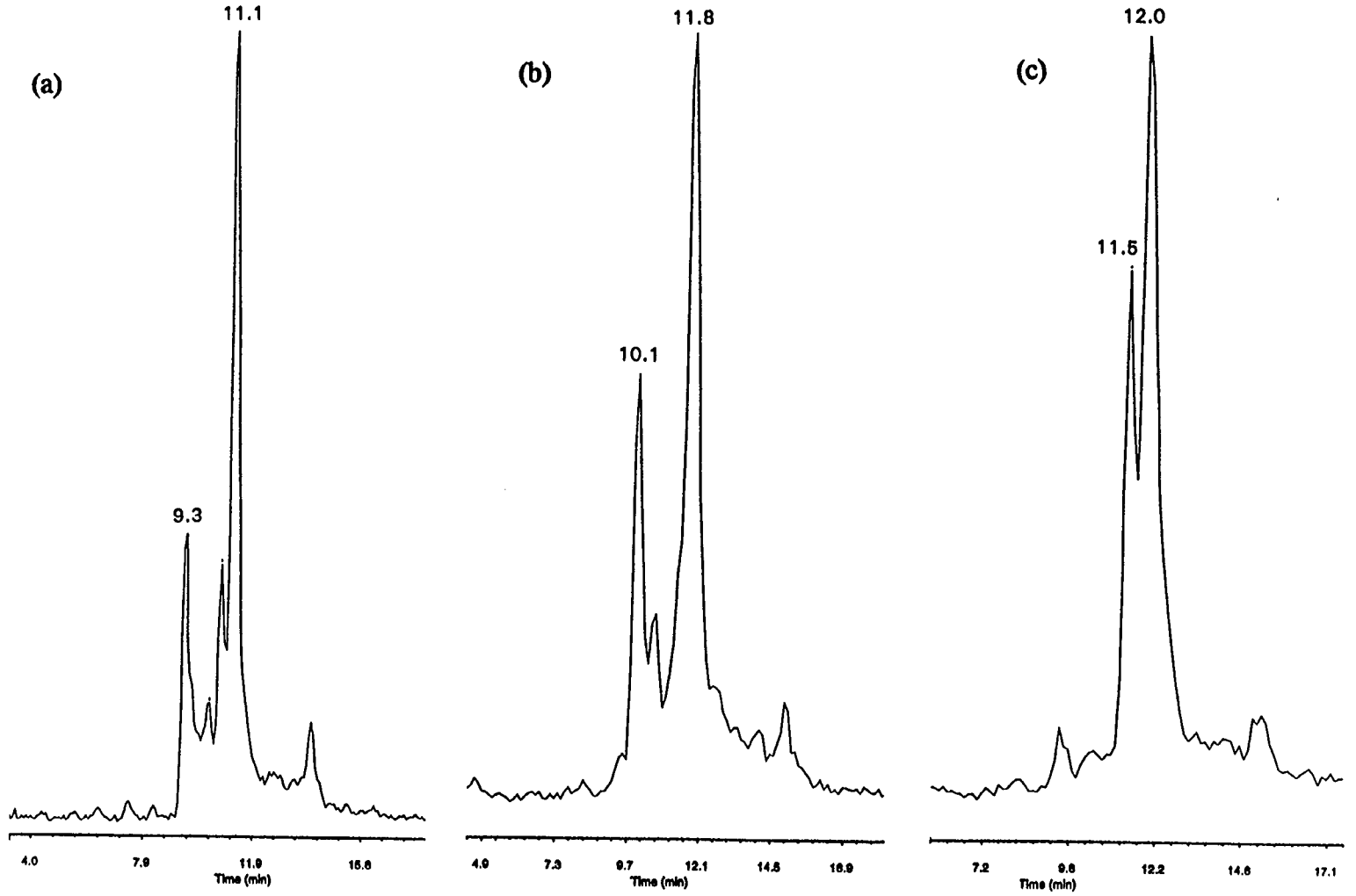


Figure 5.3 TIC chromatograms for crude samples from cyclizations using (a) BOP, (b) HATU, and (c) HAPyU.

Table 5.1 Deconvoluted molecular ions from HPLC-ion spray mass spectrometry for the crude peptide obtained from BOP cyclization

t_R (min)	Masses found[a]	Rel. Intensity (%)	Compound Mass (found)	Compound Mass[b,c] (calc.)
9.3	545.3 (+3) / 817.5 (+2)	28.9	1632.8	1633.0 (**1**)
9.9	539.3 (+3) / 808.5 (+2)	4.4	1614.8	1615.0 (**3**)
10.7	539.3 (+6) / 646.9 (+5) / 808.5 (+4) /			
	1077.6 (+3) / 1615.4 (+2)	16.3	3229.5	3230.0 (**8**)
11.1	539.3 (+3) / 808.5 (+2)	35.6	1614.8	1615.0 (**2**)

[a] Charges are shown in parentheses.
[b] Calculated from monoisotopic mass.
[c] Tentative identification of the peptides are shown in parentheses.

Table 5.2 Deconvoluted molecular ions from HPLC-ion spray mass spectrometry for the crude peptide obtained from HATU cyclization

t_R (min)	Masses found[a]	Rel. Intensity (%)	Compound Mass (found)	Compound Mass[b,c] (calc.)
10.1	578.3 (+3) / 866.8 (+2)	24.4	1731.6	1732.1 (**4**)
10.7	429.0 (+4) / 572.3 (+3) / 857.8 (+2)	7.4	1713.1	1714.1 (**6**)
11.3	808.8 (+4) / 1078.3 (+3)	8.3	3231.4	3230.0 (**8**)
11.5	559.3 (+6) / 670.5 (+5) / 838.0 (+4)			
	1117.0 (+3)	14.1	3348.2	3347.1 (**9**)
11.8	405.0 (+4) / 539.5 (+3) / 809.0 (+2)	39.0	1615.8	1615.0 (**2**)

[a] Charges are shown in parentheses.
[b] Calculated from monoisotopic mass.
[c] Tentative identification of the peptides are shown in parentheses.

Table 5.3 Deconvoluted molecular ions from HPLC-ion spray mass spectrometry for the crude peptide obtained from HAPyU cyclization

t_R (min)	Masses found[a]	Rel. Intensity (%)	Compound Mass (found)	Compound Mass[b,c] (calc.)
11.5	447.0 (+4) / 595.5 (+3) / 893.0 (+2)	27.7	1783.8	1784.0 (**5**)
11.9	442.3 (+4) / 589.5 (+3) / 883.8 (+2)	5.6	1765.3	1766.0 (**7**)
12.0	405.0 (+4) / 539.5 (+3) / 809.0 (+2)	42.9	1615.8	1615.0 (**2**)
12.3	341.0 (+10) / 379.3 (+9) / 567.5 (+6)			
	680.8 (+5) / 851.0 (+4) / 1134.3 (+3)	14.5	3400.0	3398.1 (**10**)

[a] Charges are shown in parentheses.
[b] Calculated from monoisotopic mass.
[c] Tentative identification of the peptides are shown in parentheses.

Similar to the cyclization reaction using BOP reagent, there were several peptide products observed from the cyclization reaction using HATU reagent (Figure 5.3b and Table 5.2). The two major peptide products with retention times of 10.1 and 11.8 min had molecular masses which corresponded to the Tmg-linear peptide, **4**, and the cyclic peptide, **2**, respectively. These two peptides were identical products to those isolated following cyclization with HBTU (see chapter 4). An additional peak with a mass of 1615.8 at t_R = 10.1 min could be due to a fragmentation of **4** at the β,γ-bond on the modified side chain of Dap(Tmg) which was easily cleaved in ion spray MS (see below). The peptide at t_R = 10.7 min had a mass that was 18 less than the Tmg-linear peptide, **4**, again suggesting an aspartimide peptide **6**, derived in this case from the Tmg-containing peptide. Dimeric peptides were observed in the shoulder peak eluting at 11.3 - 11.5 min. They appeared to be the cyclodimeric peptide **8**, and the Tmg linear dimer **9**.

The HPLC profile obtained following the cyclization with HAPyU was also complicated with various peptide products with close retention times (Figure 5.3c and Table 5.3). The peak at 11.5 min exhibited molecular masses of 1783.8 and 1616.4. Similar to the observation for the crude peptide from the HATU cyclization, the molecular mass of 1783.8 appears to be the alkylated peptide resulting from transfer of the dipyrrolidinyluronium group from HAPyU to the free amine side-chain of Dap to give the linear peptide [D-Asp^2,Dap(Dpg)^5]Dyn A-(1-13)NH_2, **5** (Dpg = dipyrrolidinylguanidinium); the mass of 1616.4 could be the same ion observed for the Tmg-containing peptide **4** resulting from fragmentation (β,γ-bond cleavage) of the

modified side-chain of Dap. The area where the peaks at 11.5 and 12.0 min overlapped showed multiply charged ions for a peptide with molecular mass of 1765.3. This mass could also result from aspartimide rearrangement of the Dpg-containing peptide **5**. The largest chromatographic peak at t_R 12.0 min had a mass corresponding to the cyclic peptide, **2**, and the late shoulder of this peak was tentatively identified as the Dpg linear dimer, **10**.

Ion spray tandem mass spectrometry was used to verify the structure of several peptide products and characterize fragmentation patterns for the cyclic peptide and several linear byproducts. The ion spray-tandem mass spectrum of the precursor ion 818 (+2) from the purified linear peptide **1** yielded a number of useful fragment ions (Figure 5.4). Characteristic peaks at an *m/z* of 1117 (w_9) and 1100 (w_9-NH_3) were obtained from fragmentation of the β,γ-bond of the Dap side-chain. This observation was also confirmed by the presence of doubly charged ions at *m/z* 559 and 551. In addition, a series of singly charged ions y_n were found from the C-terminus (y_1) through Arg^6 (y_8), characteristic of a peptide containing several positively charged residues at the C-terminus [Biemann, 1988]. Some doubly charged ions for y_{11} and z_{11}-NH_3 were also observed. The series of a_n and b_n were observed for N-terminal fragments through Gly^3 or Phe^4. The tandem mass spectra obtained from *m/z* 867(+2) and 893(+2) for the Tmg and Dpg linears **4** and **5** did not provide as much information as observed for peptide **1**. Similar patterns for all three linear peptides were observed, however, particularly the ions at *m/z* 551, 559, 800, 809, 1100 and 1117 (Figure 5.5). For peptide **4**, an ion corresponding to the mass of

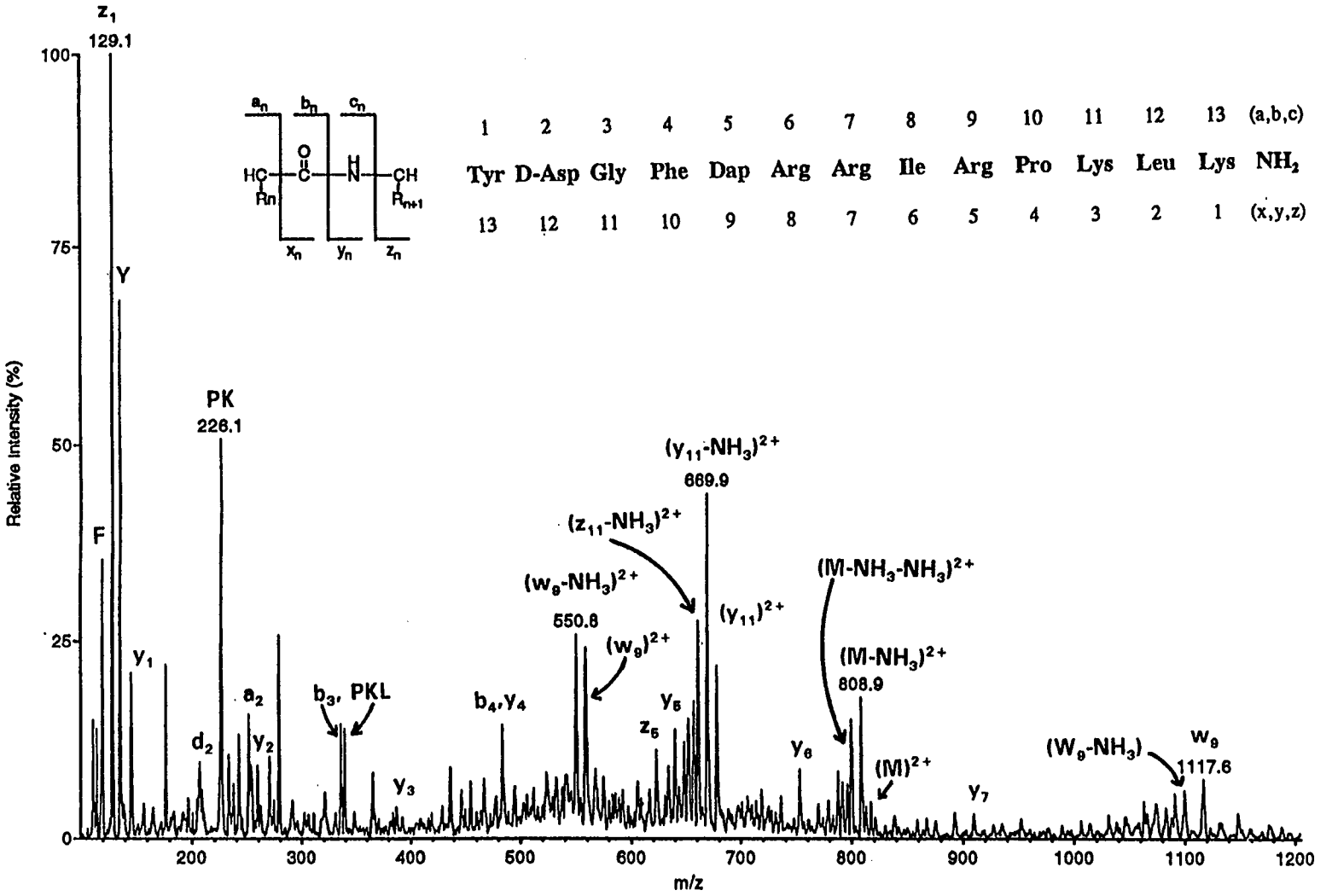


Figure 5.4 Ion spray tandem mass spectrum for the linear peptide 1 with *m/z* 818 selected as the precursor ion.

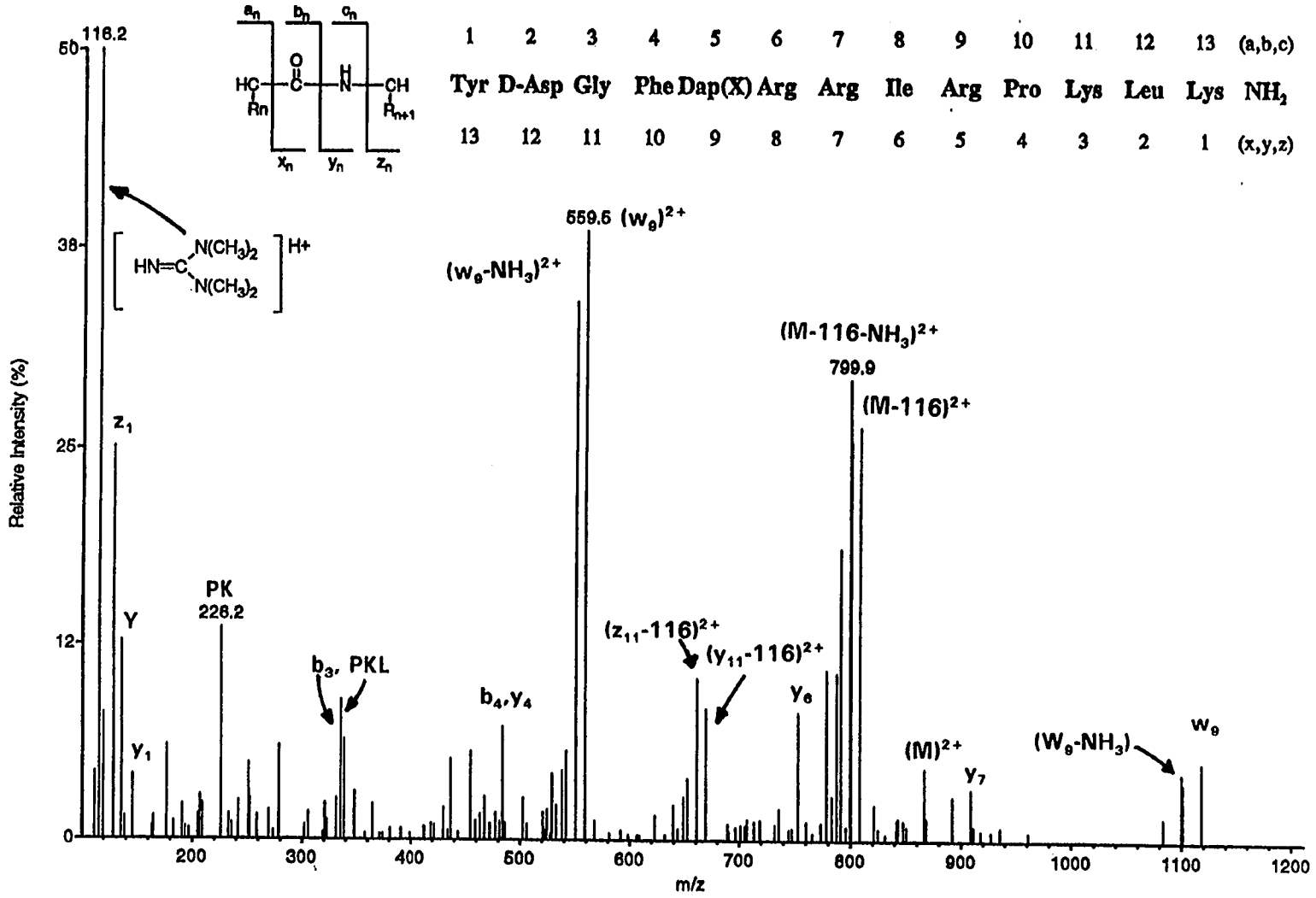


Figure 5.5 Ion spray tandem mass spectrum for the Tmg linear peptide 4 with *m/z* 867 selected as the precursor ion.

tetramethylguanidinium ion was observed at *m/z* 116 (Figure 5.5) and for peptide **5** the dipyrrolidinylguanidinium ion was found at *m/z* 168 (data not shown). The ions at *m/z* 551, 559, 1100, 1117 and 116 or 168 corresponded to the cleavage at β,γ-bond of the Dap side-chain (Figure 5.6). This cleavage is characteristic of the Dap side-chain since the tandem mass spectrum of [D-Asp^2,Dab(Tmg)^5]Dyn A-NH_2 did not show any evidence for this particular cleavage (data not shown). The ions at *m/z* 800 and 809 which were identified as ($[M-116-NH_3]^{2+}$) and ($[M-116]^{2+}$) for peptide **4** or ($[M-168-NH_3]^{2+}$) and ($[M-168]^{2+}$) for peptide **5** also support the cleavage of β,γ-bond of the Dap side-chain.

Ion spray tandem mass spectrometry was also performed to determine whether the peptide of molecular mass 1713.1 could result from aspartimide rearrangement of **4**. Due to the small amount of peptide found in the crude sample from the HATU cyclization, the ion intensity of *m/z* 858(+2) was low. The tandem mass spectrum (Figure 5.7) was slightly different form that of the Tmg linear peptide, **4** (Figure 5.6). The absence of doubly charged ions 661 ($[z_{11}-116]^{2+}$) and 670 ($[y_{11}-116]^{2+}$), and the doubly charged ions 791 ($[M-116-NH_3]^{2+}$) and 800 ($[M-116]^{2+}$) having *m/z* 9 less than those found in the tandem spectrum of the Tmg linear peptide, **4** provides supporting evidence of an aspartimide rearrangement.

Sequencing of the cyclic peptide **2** using ion spray tandem mass spectrometry is shown in Figure 5.8. Since a low energy (30 eV) was used for collision induced dissociation, fragment ion products containing an intact ring (*m/z* 505.8, 523 (a_5) and 550.8 (b_5)) were observed (see Figure 5.9). The linear portion could be sequenced

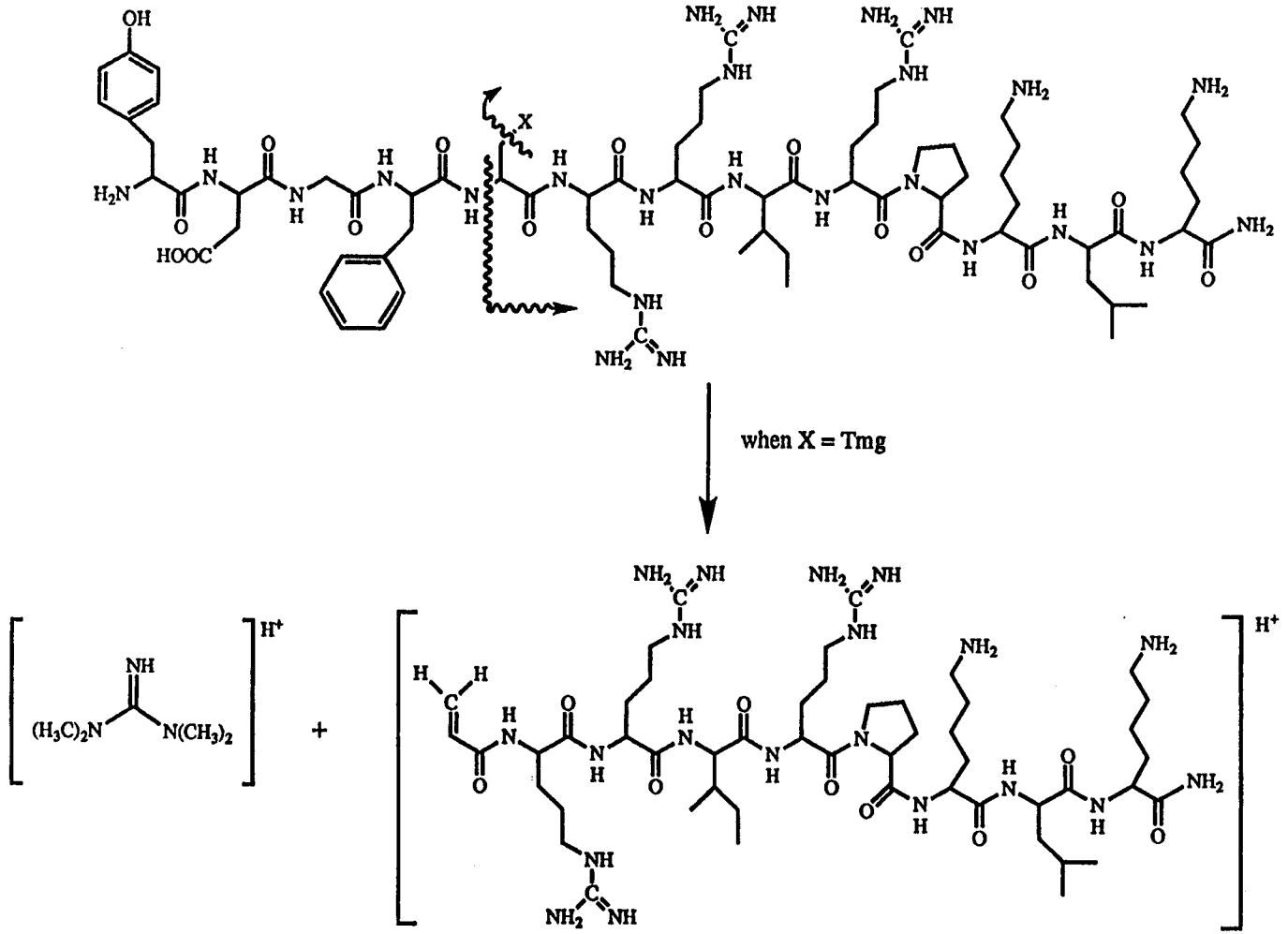


Figure 5.6 Scheme for fragmentation of the β,γ-bond cleavage on the Dap side chain giving a w_9 fragment.

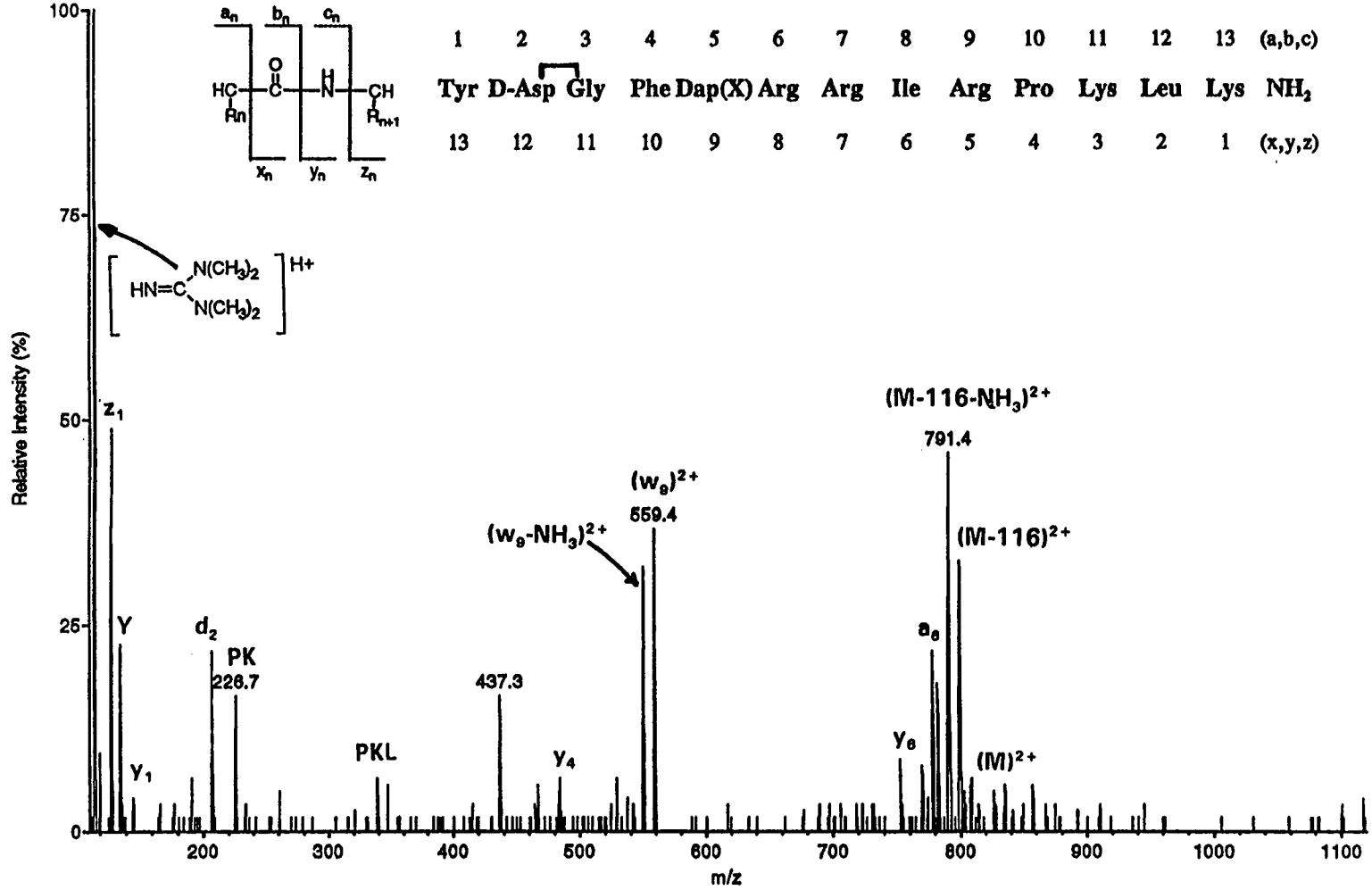


Figure 5.7 Ion spray tandem mass spectrum for the Tmg imide peptide 6 with *m/z* 858 selected as the precursor ion.

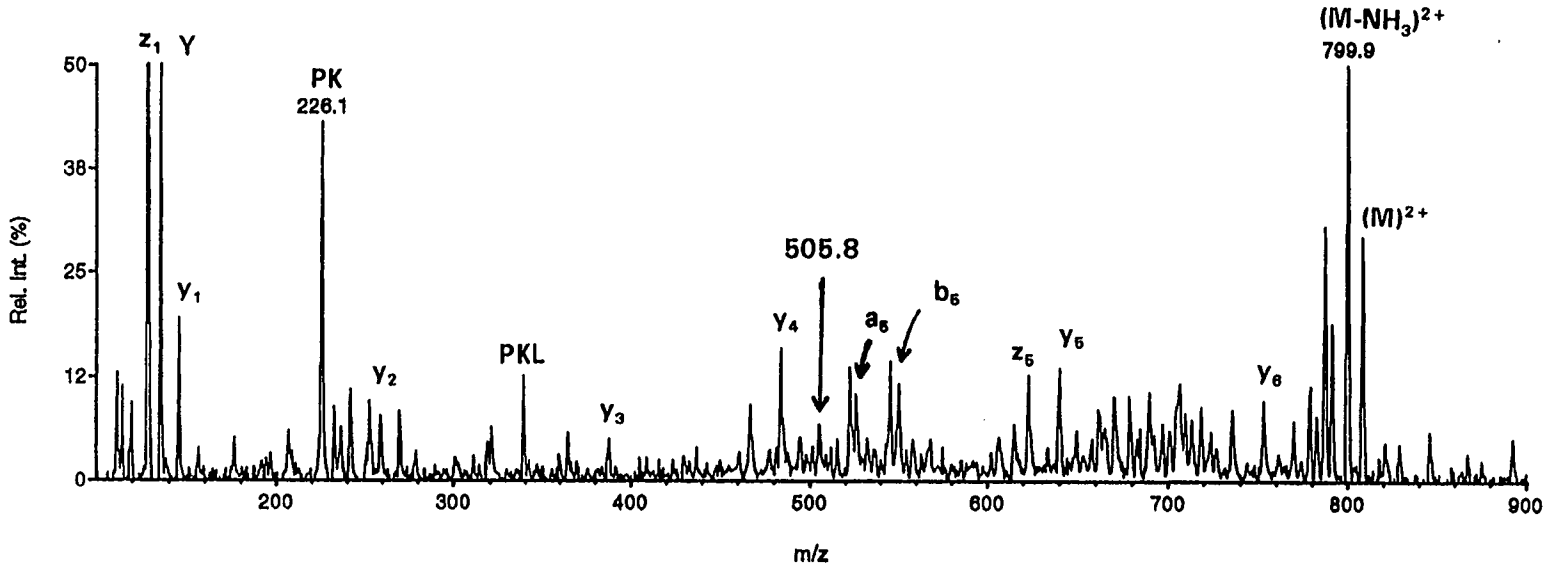


Figure 5.8 Ion spray tandem mass spectrum for the cyclic peptide 2 with *m/z* 808 selected as the precursor ion.

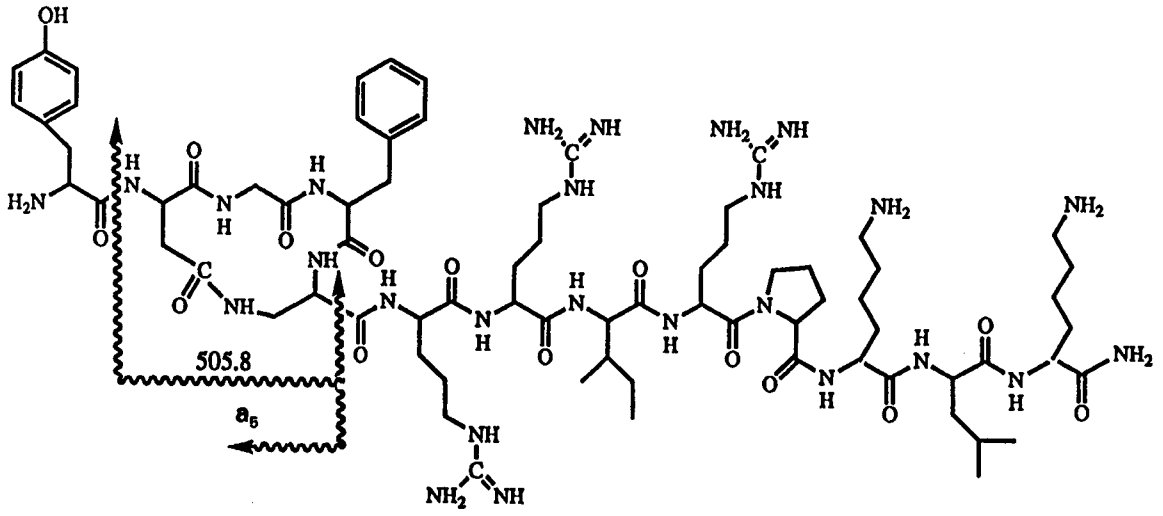


Figure 5.9 Scheme for fragmentation of cyclic peptide 2 to give fragments containing the ring portion.

from abundant ion products of the y_n type; the series of singly charged ions y_1 (C-terminus) to y_6 were observed (Figure 5.8).

5.5 Conclusions

In our study, the conventional HPLC method could not differentiate some peptide byproducts due to their similar hydrophobic properties. On-line HPLC-ion spray mass spectrometry therefore was a valuable technique to solve this problem. The difference in molecular masses observed under a peak eluting at the same retention time provided evidence that more than one peptide existed. Using this technique, we could examine several peptide byproducts from cyclizations using different activating reagents. By comparing molecular masses obtained from ion spray mass spectrometry to the theoretical values, these peptide byproducts were tentatively assigned as: the Tmg linear peptide, the Tmg linear peptide imide, the cyclodimer and the Tmg linear dimer from the cyclizations using HBTU or HATU; the Dpg linear peptide, the Dpg linear peptide imide and the Dpg linear dimer from the cyclization using HAPyU; the linear peptide, the linear peptide imide and the cyclodimer from the cyclization using BOP.

Ion spray tandem mass spectrometry is a useful technique that was used to confirm peptide structures. The sequence information could be obtained from either crude or pure peptides. Although the sequencing information was not complete, comparison of spectra of the modified peptide byproducts and the known peptide allowed tentative identification of the byproduct structures. In this study, the Dap side-chain exhibited characteristic fragmentation of the β,γ-bond. This cleavage was

observed for the side-chain of Dap as well as the modified side-chain derivatives. The ions found at *m/z* 116 and 168 were therefore identified to be the tetramethylguanidinium and dipyrrolidinylguanidinium fragments, respectively. These fragments were very useful for characterization of Dap(Tmg) or Dap(Dpg) containing peptides.

CHAPTER 6

SYNTHESIS AND OPIOID ACTIVITY OF CONFORMATIONALLY CONSTRAINED DYNORPHIN A ANALOGUES: PART I. CONFORMATIONAL CONSTRAINT IN THE "MESSAGE" SEQUENCE

Seksiri Arttamangkul[1], Thomas F. Murray[1], Gary E. DeLander[1], and Jane V. Aldrich[1,2]

A preliminary report of this work was presented at the 203rd American Chemical Society National Meeting, San Francisco, CA, April 5-10, 1992, MEDI 74. *Abstr. Papers Am. Chem. Soc.* **1992**, *203*, 74. This manuscript has been submitted to *Journal of Medicinal Chemistry*.

[1] College of Pharmacy
[2] Center for Gene Research and Biotechnology

Abbreviations:

Abbreviations used for amino acids follow the rules of the IUPAC-IUB Joint Commission of Biochemical Nomenclature in *Eur. J. Biochem.* **1984**, *138*, 9-37. Amino acids are in the L-configuration except when indicated otherwise. Additional abbreviations used are as follows: Boc, *tert*-butyloxycarbonyl; CD, circular dichroism; ClZ, 2-chlorobenzyloxycarbonyl; Dab, α,γ-diaminobutyric acid; Dap, α,β-diaminopropionic acid; DAMGO, [D-Ala^2,MePhe^4,Gly-ol]enkephalin; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DMA, *N,N*-dimethylacetamide; DMF, *N,N*-dimethylformamide; DPDPE, cyclo[D-Pen^2,D-Pen^5]enkephalin; Dyn A, dynorphin A; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, (9-fluorenylmethoxy)carbonyl; Fmoc-OSu, 9-fluorenylmethyl succinimidyl carbonate; GPI, guinea pig ileum; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MBHA, 4-methylbenzhydrylamine; NMR, nuclear magnetic resonance; PAL® resin, Peptide Amide Linker or 5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid resin; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl, TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; THF, tetrahydrofuran; Tmg, tetramethylguanidinium; Tos, 4-toluenesulfonyl; Z, benzyloxycarbonyl.

6.1 Abstract

A constrained analogue of the opioid peptide dynorphin A (Dyn A) cyclized in the "message" sequence was designed which may be compatible with the helical conformation proposed by Schwyzer (*Biochemistry* **1986**, *25*, 4281-4286) as the conformation Dyn A adopts at κ opioid receptors. Based on molecular modeling with AMBER, we prepared the lactam cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2 (**1**, Dap = α,β-diaminopropionic acid) containing a four-atom bridge between positions 2 and 5 as a possible constraint compatible with an α-helix, along with the homologues with five- (**2**) and six-atom (**3**) bridges containing Dab (α,γ-diaminobutyric acid) and Orn, respectively, in position 5. All of the cyclic peptide analogues exhibited high binding affinity for both κ and μ receptors and high potency in the guinea pig ileum (GPI) assay. As ring size increased, a trend in receptor selectivity from slightly κ selective (compound **1**) to nonselective for κ vs. μ (compound **2**) to slightly μ selective (compound **3**) was observed. The results in the GPI for antagonism of these peptides by naloxone paralleled the results of the binding assays and indicated that compound **1** preferentially interacted with κ receptors in this tissue. Novel byproducts were also obtained from the cyclization reactions with HBTU (*O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and characterized as [D-Asp^2,X(Tmg)^5]Dyn A-(1-13)NH_2 (where X = Dap, Dab or Orn and Tmg = tetramethyguanidinium). All of the Tmg linear byproducts bound with high affinity to κ and μ receptors and also exhibited good activity in the GPI. Circular dichroism spectra for compound **1** and the parent peptide Dyn A-(1-13)NH_2 determined in 80% trifluoroethanol at 5°C were

consistent with some α helical content in the peptides; the elipticity at 222 nm suggested that compound **1** possessed slightly higher helical content than Dyn A-(1-13)NH_2 under these conditions. The similarities in κ receptor affinity, potency in GPI and the secondary structure observed from CD of compound **1** and Dyn A-(1-13)NH_2 suggested that compound **1** is capable of adopting a conformation similar to a biologically active conformation of Dyn A-(1-13)NH_2.

6.2 Introduction

Since their discovery, considerable research has focused on understanding the physiological and pharmacological roles of opioid receptors. Establishment of clear-cut relationships between specific opioid receptors and their distinct opioid effects has been complicated, however, by the existence of multiple forms of both opioid receptors and their endogenous ligands [Simon and Hiller, 1994]. Three opioid receptor types, the μ, δ and κ receptors, are generally accepted and have recently been cloned [Kieffer et al., 1992; Evans et al., 1992; Yasuda et al., 1993; Chen et al., 1993]. The endogenous mammalian opioid peptides are also classified into three families, the enkephalins, the dynorphins and the endorphins [Simon and Hiller, 1994]. Although enkephalins have high affinity for δ receptors, dynorphins for κ receptors and endorphins for μ receptors, each of these ligands has significant affinity for more than one receptor type [Simon, 1991]. One goal in opioid research is the development of ligands highly selective for each receptor type, since these ligands can be both potential therapeutic agents and valuable pharmacological tools for understanding the biological effects produced by different receptors. During the last

decade considerable effort has focused on the development of κ selective opioid agonists as potential analgesics without the significant clinical side-effects associated with morphine and other μ receptor selective analgesic drugs [Millan, 1990; Rees, 1992]. We are interested in developing highly potent and selective ligands for κ receptors as pharmacological tools to study opioid receptor structure and function using the endogenous opioid peptide, dynorphin A, as the prototype.

Dynorphin A (Dyn A), a 17-amino acid peptide, is postulated to be an endogenous ligand for κ opioid receptors [Chavkin et al., 1982]. Dyn A has an identical N-terminal tetrapeptide sequence (the "message" sequence) [Chavkin and Goldstein, 1981] to other mammalian opioid peptides, and exhibits remarkably potent opioid activity [Goldstein et al., 1979]. Dyn A-(1-13), the peptide fragment first reported, is about 700 times more potent than [Leu]enkephalin and about 50 times more potent than β-endorphin in the guinea pig ileum (GPI) assay. Its unusually high potency is believed to result from the C-terminal sequence (residues 6-13, the "address" sequence) which directs this peptide to κ receptors [Chavkin and Goldstein, 1981]. Dyn A-(1-13) exhibits a similar receptor binding profile to Dyn A [Corbett et al., 1982], and therefore Dyn A-(1-13) has often served as the parent structure for developing analogues with improved κ selectivity. Several studies have examined Dyn A fragments and amino acid replacement to determine the relative importance and structure-activity relationships for individual residues of Dyn A [Chavkin and Goldstein, 1981; Turcotte et al., 1984; Snyder et al.; 1992; Story et al., 1992; Kawasaki et al., 1993b].

Dyn A is a highly flexible molecule which is capable of assuming a number of different conformations, and the biologically active conformations of this peptide are still unclear. This conformational flexibility may be one reason for the peptide's low κ receptor selectivity. Conformational constraint is one approach which can be used not only to restrict the flexibility of the peptide molecule, but also to provide information on the topographical requirements of receptors [Hruby et al., 1990]. In addition, highly potent and selective ligands may be found when appropriate conformational constraints are incorporated.

Conformational constraint by cyclization has been successfully employed in the development of several potent opioid peptide analogues such as the potent and highly selective μ and δ receptor agonists, H-Tyr-cyclo[D-Orn-Phe-Asp]-NH_2 [Schiller et al., 1985] and DPDPE (cyclo[D-Pen^2,D-Pen^5]enkephalin) [Mosberg et al., 1983]. Several cyclic Dyn A analogues have also been synthesized and evaluated for their biological activity, but studies of constrained Dyn A analogues have been far less extensive than those reported for μ- and δ-selective peptides. Only two Dyn A analogues have been reported with a constraint in the "message" sequence of the peptide. A disulfide bridge between D-Cys in position 2 and L-Cys in position 5 produced a peptide which is 5-fold more potent than the parent 13-mer in the GPI assay [Schiller et al., 1982], but tolerance studies suggested that it interacted mainly with δ receptors and had only minor interactions with κ receptors [Shearman et al., 1985]. A cyclic lactam cyclo[D-Orn^2,Asp^5]Dyn A-(1-8)NH_2 has also been prepared, but the high sensitivity of this peptide to antagonism by naloxone in the GPI (K_e =

1.49 nM) suggests that this peptide preferentially interacts with μ receptors in this tissue [Schiller et al., 1988]. Other cyclic analogues have involved modifications in the "address" segment [Schiller et al., 1988; Kawasaki et al., 1990; Kawasaki et al., 1993a]. Several cyclic disulfide analogues exhibited unexpected selectivities for the receptors in the central vs. peripheral nervous system, which suggested possible receptor differences between these sites [Kawasaki et al., 1990; Kawasaki et al., 1993a]. The cyclic lactam analogues of Dyn A, cyclo[Orn^5,Asp^8]Dyn A-(1-13)NH_2, cyclo[Orn^5,Asp^{10}]Dyn A-(1-13)NH_2 and cyclo[Orn^5,Asp^{13}]Dyn A-(1-13)NH_2, have also been prepared [Schiller et al., 1988]. These analogues showed high affinity for μ receptors, however, and antagonism of their opioid activity in the GPI by low doses of naloxone suggested that these compounds did not interact significantly with κ receptors.

Using equilibrium thermodynamic and kinetic estimations, Schwyzer proposed a helical conformation for the N-terminus of Dyn A-(1-13) oriented perpendicular to the membrane surface [Schwyzer, 1986]. The α-helix was proposed to extend from Tyr^1 through Arg^9 with the C-terminal residues Pro^{10} to Lys^{13} in an unordered structure. We were interested in stabilizing this suggested helical structure of Dyn A through a lactam linkage. From our preliminary study of possible conformational constraints using the molecular modeling program AMBER [Weiner and Kollman, 1981; Weiner et al., 1984; Weiner and Kollman, 1986], we found that a four-atom bridge containing an amide bond between residues *i* (D-configuration) and *i+3* (L-configuration) appeared to be ideal for spanning the distance between C_i^{α} and C_{i+3}^{α} in

an α-helix. We were interested in incorporating this constraint into the "message" sequence and therefore prepared the cyclic analogue cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2 (**1**, Dap = α,β-diaminopropionic acid) containing the four atom bridge between the α carbons of residues 2 and 5. In addition, the analogues with five- (**2**) and six-atom bridges (**3**) were synthesized by incorporating Dab (α,γ-diaminobutyric acid) and Orn, respectively, into position 5 of the peptides in order to examine the effect on opioid activity and receptor affinity of varying the bridge length (see Figure 6.1).

During the formation of these relatively small (14- to 16-membered) lactam rings using HBTU, we obtained another major peptide product in addition to the desired cyclic peptides. We evaluated the cyclic peptides, along with the unusual linear byproducts obtained from the cyclization reactions, for their affinity for κ, μ and δ opioid receptors and for their opioid activity in the GPI assay. The results of these assays are described below.

6.3 Experimental

6.3.1 Materials

Peptide syntheses were performed by solid phase synthesis using a Milligen Biosearch 9500 automated peptide synthesizer (Novato, CA), and HF cleavages performed on a Multiple Peptide Systems HF Apparatus model 2010C (San Diego, CA). Reverse phase HPLC was done on a Beckman model 431A gradient system. The analytical HPLC columns were a Dupont Zorbax Protein Plus analytical column

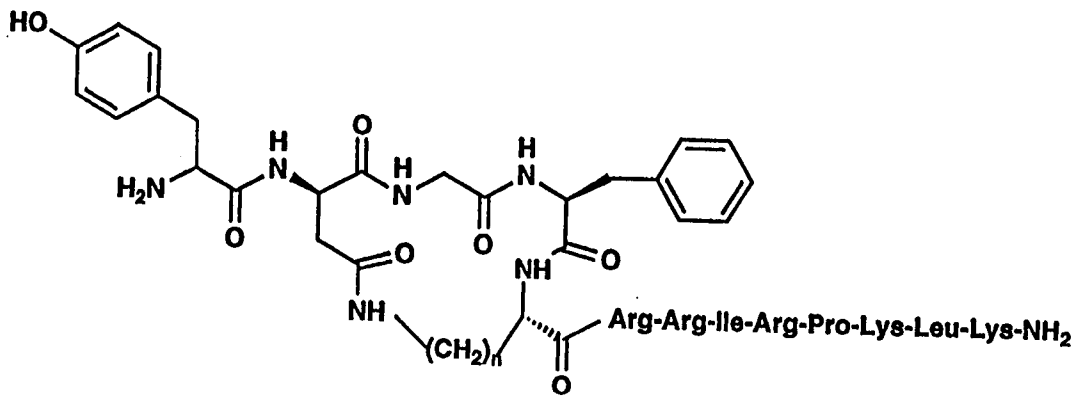


Figure 6.1 Cyclic dynorphin A analogues

(C_3, 300 Å, 10μ , 4.6 mm x 25 cm) equipped with a Protein Plus guard cartridge and a 214TP54 Vydac analytical column (C_4, 300 Å, 5 μ, 4.6 mm x 25 cm). Preparative reversed phase HPLC was performed on a Rainin gradient HPLC system using a Dupont Zorbax Protein Plus preparative column (C_3, 300Å, 10μ, 21mm x 25cm) with a Dynamax (C_4, 12μ, 21mm x 5cm) guard cartridge. Amino acid analyses were performed after hydrolysis of the peptides under standard conditions (6N HCl plus 1.0% phenol at 110°C for 20 h). Each amino acid was resolved using a step gradient on a Beckman Spherogel 52C ion exchange column (4.6 x 15 cm) equipped with post-column ninhydrin detection on a Beckman 126AA System Gold HPLC amino acid analyzer. Peptide sequencing was performed via Edman degradation on an Applied Biosystems, Model 475A gas-phase protein sequencer. Both amino acid analysis and peptide sequencing were performed by the Central Services Laboratory, Center for Gene Research and Biotechnology, Oregon State University. Molecular weights of the peptides were determined by FAB mass spectrometry in the positive mode on a Kratos MS50RFTC in the Department of Agricultural Chemistry, Oregon State University. NMR spectroscopy was performed on a 300 MHz Bruker NMR in the Department of Chemistry, Oregon State University. Elemental analysis was performed by MWH Laboratories, Phoenix, AZ.

6.3.2 Amino Acid Synthesis: N^{α}-Fluorenylmethoxycarbonyl-N^{β}-t-butoxycarbonyl-L-α,β-diaminopropioic acid (10)

N^{α}-Benzyloxycarbonyl-L-asparagine (Bachem) was rearranged to N^{α}-benzyloxy-carbonyl-L-α,β-diaminopropioinic acid (56% yield) using [I,I-

bis(trifluoroacetoxy) iodo]-benzene (Aldrich) as reported in the literature [Waki et al., 1981]. The crystalline ZDap obtained was then protected with Boc, as previously described [Bodanszky and Bodanszky, 1984], using *t*-butyl pyrocarbonate (Aldrich) to give N^{α}-benzyloxycarbonyl-N^{β}-*t*-butyloxycarbonyl-L-α,β-diaminopropioinic acid (75% yield). The Z protecting group was removed by catalytic transfer hydrogenation using 10% palladium on carbon (Aldrich) and ammonium formate (Aldrich) [Anwer and Spatola, 1980], and then the Fmoc protecting group introduced at the α-amine using Fmoc-OSu (Bachem, 0.9 equiv) plus Na_2CO_3 (1 equiv) in THF/H_2O (1/1) [Snyder et al., 1993]. Crude **10** was purified by medium pressure liquid chromatography on silica gel (grade 60, 230-400 mesh, 60 Å, Merck 9385) using first DCM followed by DCM/MeOH/AcOH (95/5/0.1) at a flow rate of approx. 10 mL/min. Purified **10** was precipitated from MeOH with H_2O to yield a fine white powder (87% yield): mp 97-100°C; FAB-MS *m/z* 425.1(M-1); TLC R_f (DCM/MeOH/AcOH, 85/10/5) 0.77; ^{1}H-nmr (300 MHz, MeOH-d_4) δ 1.45 (s, 9H, -C(C$\underline{H}_3$)$_3$), 3.32 (m, 2H, βCH_2), 4.25 (m, 2H, αCH and Fmoc-C$\underline{H}$CH$_2$O), 4.34 (d, 2H, Fmoc-CHC$\underline{H}_2$O), 7.34 (m, 4H, Ar), 7.66 (d, 2H, Ar), 7.79 (d, 2H, Ar); $[\alpha]_D^{25}$ -4.3° (c 1.0, MeOH). Anal. ($C_{23}H_{26}N_2O_6 \cdot H_2O$): C, H, N.

6.3.3 Cyclic Peptide Synthesis

For syntheses of the cyclic peptides, Fmoc-amino acids (Bachem, Novabiochem and Bachem Biosciences (FmocDab(Boc))) were assembled on an MBHA resin (Bachem, 0.23 mmol/g, 1% cross-linked polyvinyl styrene) using the HBTU (Amino Tech) coupling protocol as previously described [Story and Aldrich

1994]. After the last amino acid was attached, the *t*-butyl side chain protecting groups were removed with 50% (v/v) TFA plus 2% anisole in DCM (5 min and then 30 min). Following neutralization with 10% (v/v) DIEA in DCM (3 x 30 mL) and washing with DCM (5 x) and DMA/DCM (1/1, 5 x), cyclizations were carried out at room temperature by the addition of HBTU/HOBt (4.0 equiv each) in DMF containing excess DIEA (6.0 equiv). The reaction was usually complete within 2 - 3 h, as determined by the Kaiser test. The N-terminal Fmoc groups were then removed using 30% piperidine in DMA/toluene (1/1), and the peptide resins washed with DMA/DCM (1/1, 10 x), DCM (6 x) and MeOH (4 x) and dried *in vacuo* over night. The peptides were cleaved from the resin by treatment with liquid HF (10 mL of HF plus 1 mL of anisole/g of resin) for 60 min at 0° C. After evaporation of HF, the resin was extracted with Et_2O (3 x 10 mL) and then with 0.5 N AcOH (3 x 10 mL). The crude peptides were obtained by lyophilization of the aqueous extracts.

Peptides were desalted by gel filtration through a Sephadex G-10 column (2.6 x 16.3 cm) using 0.5 N AcOH. Following lyophilization the crude peptides were purified further by reverse phase HPLC using a mobile phase gradient of 20-50% MeOH in 0.1% TFA over 30 min at a flow rate of 20 mL/min. In all cases two major peaks of peptides were obtained. The pure fractions from each peak were collected and lyophilized. Homogeneity of the peptides was established by analytical HPLC using a mobile phase gradient of 0-75% AcCN in 0.1% TFA over 50 min at a flow rate of 1.5 mL/min. All peptides were > 98% pure, as judged from the HPLC elution profiles.

6.3.4 Linear Peptide Synthesis

The linear peptide **7** was synthesized by standard Fmoc protocol on a PAL® resin (Peptide Amide Linker resin, Millipore, 0.27 mmol/g) as previously described [Snyder et al., 1992]. Following cleavage of the peptide with Reagent K (82.5% TFA, 5% water, 5% phenol, 5% thioanisole, and 2.5% ethanedithiol) [King et al., 1990], the peptide was purified by reversed phase HPLC using a gradient of 0 - 50% AcCN containing 0.1% TFA in 100 min at 10 mL/min.

6.3.5 Circular Dichroism

Circular dichroism spectra were recorded on a J-720 Jasco spectrometer (Japan Spectroscopic Co. Ltd., Tokyo). The instrument was routinely calibrated with (+)-10-camphorsulfonic acid for proper operation [Chen and Yang, 1977]. The peptides were freshly prepared in aqueous buffer containing 10 mM potassium phosphate (pH 7.4) or in 80% (v/v) TFE (Aldrich) in 10 mM potassium phosphate buffer. Concentrations of the peptide solutions were determined by the guanidine hydrochloride method [Elwell and Schellmen, 1977] using absorbance of the same stock solutions at 280 nm (ε = 1280 $M^{-1}cm^{-1}$) and 190 nm. Spectra were collected at 0.5 nm intervals in the range of 178-260 nm and were the average of four runs. Spectra were smoothed using a cubic spline algorithm.

6.3.6 Receptor Binding Assays and Smooth Muscle Assays

Opioid receptor binding studies were performed as described in detail elsewhere [Story et al., 1992]. Incubations of [^{3}H]bremazocine (0.7 - 0.8 nM) with

guinea pig cerebellar membrane preparation were performed for 3 h at 4°C and incubations of rat membrane preparations with [^{3}H]DAMGO (0.3 - 0.4 nM) and [^{3}H]DPDPE (2.4 nM) were performed for 5 h at 4°C. IC_{50} values were determined by nonlinear regression analysis to fit a logistic equation to the competition data and K_i values calculated from the IC_{50} values by the Cheng and Prusoff equation [Cheng and Prusoff, 1973], using $K_D$values of 0.314, 7.63 and 0.055 nM for [^{3}H]DAMGO, [^{3}H]DPDPE and [^{3}H]bremazocine, respectively.

The GPI assays were carried out as previously described [Story et al., 1992]. A log dose-response curve was determined with Dyn A-(1-13)NH_2 (Peninsula Laboratories) as the standard for each ileum. K_e values for naloxone were determined from the ratio of IC_{50} values obtained in the presence and absence of a fixed concentration of naloxone (300 nM, added 20 min prior to addition of the agonist). IC_{50} and K_e values reported were obtained from 4 - 5 replicates in tissues from different animals.

6.4 Results and Discussion

6.4.1 Chemistry

All cyclic peptides were synthesized and cyclized on an MBHA resin. Cyclizations performed on solid phase resins utilize pseudodilution to favor intramolecular resin-bound reactions [Barany and Merrifield, 1980]. This methodology was selected in order to minimize formation of cyclodimeric or polymeric peptides which can occur when cyclizations are performed in solution, even

if the solution is very dilute [Bodanszky and Martinez, 1984]. The Fmoc group was selected for α-amine protection of the amino acids. The side chain functional groups of the amino acids involved in the cyclization reaction, D-Asp and Dap, Dab or Orn, were protected with *t*-butyl and Boc groups, respectively. The side chain protecting groups for Arg and Lys, Tos and ClZ, were chosen to be stable to TFA treatment.

The synthesis of the linear peptide **7** was performed on a PAL® resin (Peptide Amide Linker resin) using standard Fmoc synthetic strategy. The side chains of Fmoc amino acids used in this case were protected with TFA-labile functional groups, namely D-Asp(OtBu), Tyr(tBu), Lys(Boc) and Arg(Pmc).

The synthesis of the amino acid derivative FmocDap(Boc), **10**, was performed via a modified Hoffmann rearrangement. Starting with Z-Asn, the aliphatic amide was converted to an amine using [I,I-bis (trifluoroacetoxy)iodo]benzene [Waki et al., 1981], and the free amine then protected with Boc using *t*-butyl pyrocarbonate to yield ZDap(Boc) [Bodanszky and Bodanszky, 1984]. After removal of the benzyloxycarbonyl protecting group by catalytic transfer hydrogenation [Anwer and Spatola, 1980], the Fmoc protecting group was introduced on the α-amine using Fmoc-OSu [Snyder et al., 1993] to give compound **10**. An attempt to rearrange Fmoc-Asn to give Fmoc-Dap directly gave a lower yield and a product with lower purity.

The syntheses of **1-3** were performed according to the synthetic scheme shown in Figure 6.2. After incorporation of the last amino acid to the growing peptide chains, the *t*-butyl protecting groups were removed with TFA and the cyclizations

then performed on the resins using a 4-fold excess of HBTU reagent (HBTU/HOBt/DIEA, 4/4/6 equiv). The cyclization reactions all gave a negative response to ninhydrin within 2-3 hours. The N-terminal Fmoc protecting groups were removed with piperidine, and the peptide amides then liberated from the resins using liquid HF. Crude peptides were purified by gel filtration on Sephadex G-10 followed by reversed phase HPLC.

In all cases, two major peptide products were obtained. Analyses of the purified peptides using FAB-MS revealed that the desired cyclic analogues were obtained along with byproducts having molecular weights 116 higher than the corresponding cyclic peptides (Table 6.1). Amino acid analysis for the byproduct obtained from the cyclization reaction to yield **1** gave a peak which did not match Dap, and peptide sequencing produced a peak distinct from that of Dap or Asp-Dap in the fifth cycle. A similar result was observed in our laboratory during an attempt to form the highly strained 10-membered lactam ring in cyclo[Dab^2,D-Glu^3,Leu^5]enkephalinamide [Story and Aldrich, 1994]. In this case, characterization of the products using amino acid analysis, peptide sequencing, NMR and high resolution mass spectrometry indicated that the products were [$Dab(Tmg)^2$,D-Glu^3,Leu^5]enkephalinamide and the corresponding linear dimeric peptide contained the tetramethylguanidinium (Tmg) functionality, resulting from transfer of the tetramethyluronium group from HBTU to the γ-amine of Dab. This side reaction was also observed during a backbone coupling when HBTU reagent was added to the free amine solution instead of to the carboxylate solution [Gausepohl et al., 1991]. The

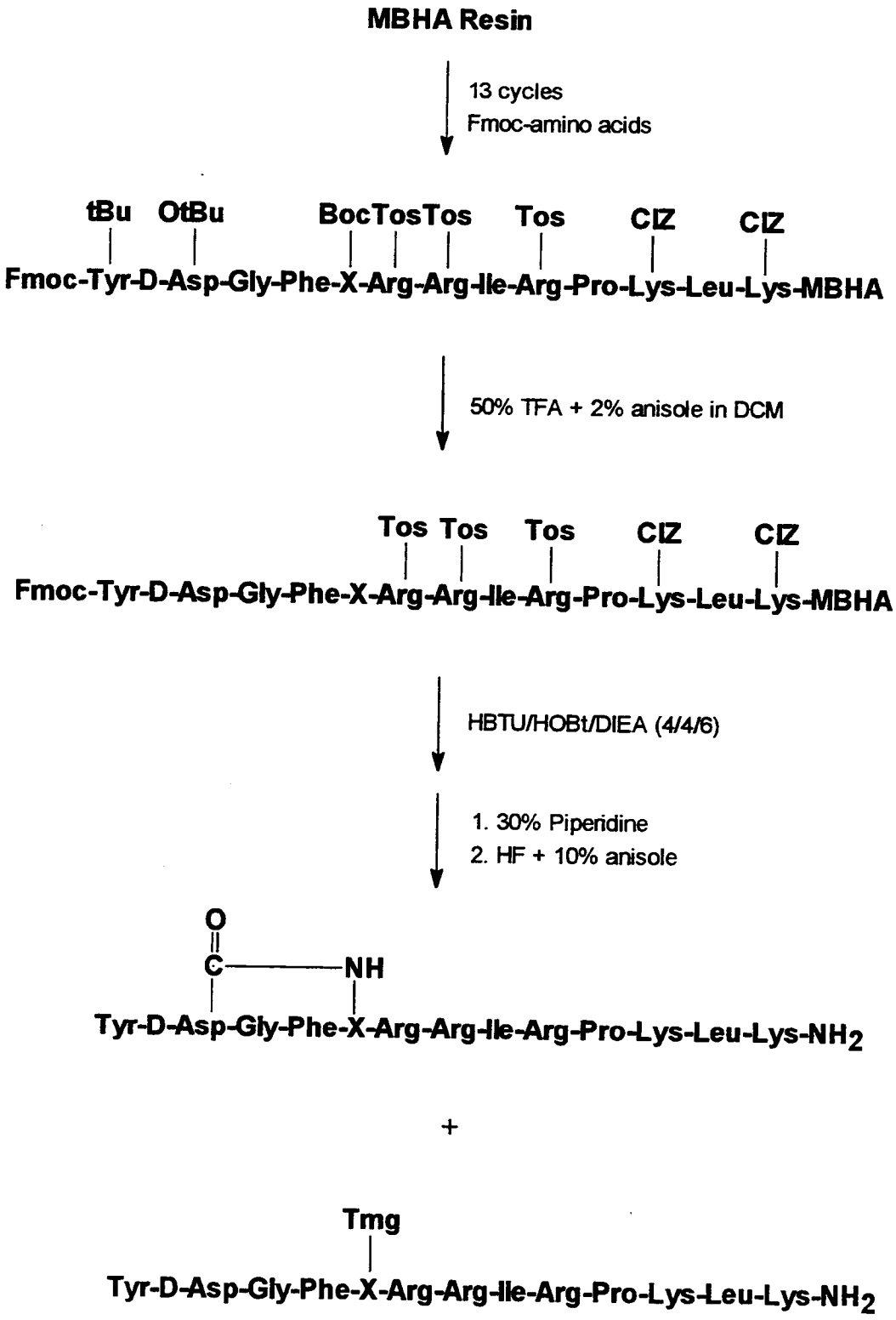


Figure 6.2 Synthesis of cyclic dynorphin A analogues

Table 6.1 Analytical Data for Dyn A-(1-13)NH_2 Analogues

Compounds	t_R[a] (min)	FAB-MS (M + 1)	Amino Acid Compositions[b]									
			Tyr (1)	Asp (1)	Gly (1)	Phe (1)	X (1)	Arg (3)	Ile (1)	Pro (1)	Lys (2)	Leu (1)
1	17.9	1615.9	0.96	1.01	0.99	1.00	0.99	3.03	0.97	0.91	2.01	1.02
2	17.1	1630.5	1.04	1.02	1.03	1.02	1.02	3.02	0.96	0.85	2.01	1.04
3	18.2	1643.9	0.79	1.06	1.16	0.86	0.96	3.12	1.03	1.02	2.00	1.04
4	15.3	1731.9	0.90	0.93	1.00	1.01	c	3.10	1.01	0.90	2.13	1.08
5	15.7	1745.8	0.89	0.91	1.00	0.99	d	3.04	1.01	0.81	2.25	1.11
6	16.2	1759.9	0.95	0.96	1.02	1.04	e	3.04	0.98	0.88	2.08	1.05
7	20.6	1661.0	1.05	1.04	1.05	1.04	-	3.14	0.90	0.76	2.00	2.01 (2)

[a] Elution as described in experimental section using the Vydac analytical column.
[b] Theoretical values are shown in parentheses.
[c] A peak was observed at 46.3 min which did not correspond to Dap (t_R = 47.8 min).
[d] A peak was observed at 46.0 min which did not correspond to Dab (t_R = 42.5 min).
[e] A peak was observed at 45.7 min which did not correspond to Orn (t_R = 40.8 min).

analytical data for the byproduct peptides obtained from the cyclization reactions of the Dyn A analogues were consistant with this identification as the linear tetramethylguanidinium derivatives **4-6** (Figure 6.3). A detailed analysis indicates that the prevalence of this side reaction is dependent upon the amino acid sequence and the reagents used for the cyclization (see chapter 4).

6.4.2 Receptor Affinity and Opioid Activity

The peptides were evaluated for their binding affinity at κ, μ and δ opioid receptors using competition binding assays (Table 6.2). κ-Receptor affinity was determined in guinea pig cerebellum, a tissue which is rich in κ binding sites [Robson et al., 1984], using [^{3}H]bremazocine as the radioligand. To measure μ and δ affinity, [^{3}H]DAMGO and [^{3}H]DPDPE were employed as selective radioligands for μ and δ receptors, respectively, in rat forebrain membrane preparations.

As summarized in Table 6.2, all of the Dyn A analogues examined exhibited high binding affinity for both κ and μ receptors ($K_i < 1$ nM), and generally had moderate binding affinity for δ receptors ($K_i = 4$ - 14 nM, except for [D-Asp^2]Dyn A-(1-13)NH_2, **7**). These results are generally similar to those observed for the parent linear peptide Dyn A-(1-13)NH_2. At κ receptors compounds **1** and **2** have binding affinity similar to that of the parent peptide. Compound **3**, which contains the largest ring size in the series, exhibited a 3- to 4.5-fold decrease in κ receptor affinity compared to **1** and Dyn A-(1-13)NH_2. Compounds **2** and **3** had similar binding affinity at μ receptors as the parent peptide, while compound **1** had 2.5-fold lower affinity for these receptors. These modest differences in affinities resulted in a trend

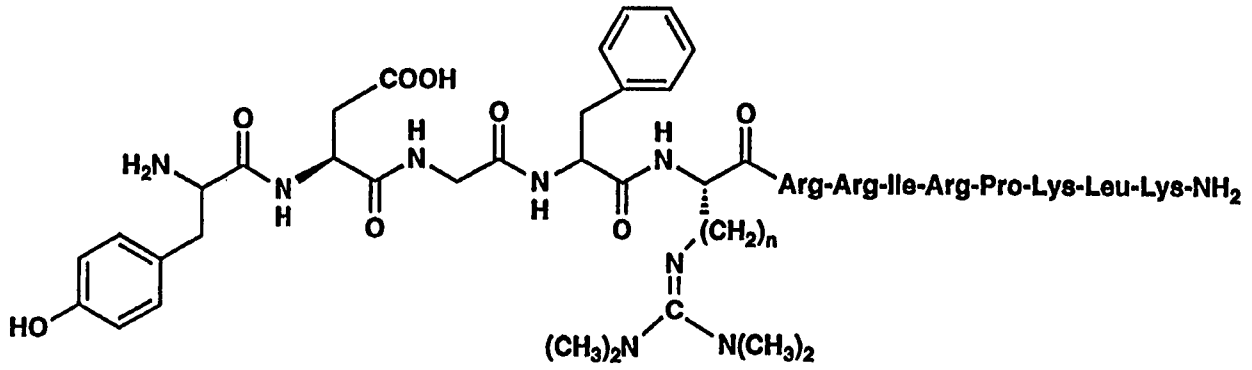


Figure 6.3 Linear tetramethylguanidinium derivatives 4 - 6

Table 6.2 Opioid Receptor Binding Affinities of Dyn A-(1-13)NH_2 Analogues

Compound No.	Analogues	K_i (nM)[a]			
		[^{3}H]Bremazocine	[^{3}H]DAMGO	[^{3}H]DPDPE	K_i Ratio ($\kappa/\mu/\delta$)
1	cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2	0.22 ± 0.002	0.49 ± 0.016	10.2 ± 0.3	1/2.2/47
2	cyclo[D-Asp^2,Dab^5]Dyn A-(1-13)NH_2	0.13 ± 0.003	0.17 ± 0.002	12.0 ± 0.4	1/1.3/92
3	cyclo[D-Asp^2,Orn^5]Dyn A-(1-13)NH_2	0.68 ± 0.033	0.19 ± 0.001	14.1 ± 0.2	3.6/1/74
4	[D-Asp^2,Dap(Tmg)^5]Dyn A-(1-13)NH_2	0.21 ± 0.003	0.16 ± 0.001	4.31 ± 0.28	1.3/1/27
5	[D-Asp^2,Dab(Tmg)^5]Dyn A-(1-13)NH_2	0.14 ± 0.005	0.32 ± 0.01	7.52 ± 0.40	1/2.3/54
6	[D-Asp^2,Orn(Tmg)^5]Dyn A-(1-13)NH_2	0.36 ± 0.011	0.28 ± 0.006	14.2 ± 0.4	1.3/1/51
7	[D-Asp^2]Dyn A-(1-13)NH_2	0.45 ± 0.07	0.013 ± 0.001	0.044 ± 0.005	35/1/3.4
8	Dyn A-(1-13)NH_2	0.15 ± 0.004	0.19 ± 0.002	3.88 ± 0.091	1/1.3/26

[a] K_i ± S.E.

in receptor selectivity from slightly κ selective (compond **1**) to non-selective (compound **2**) to slightly μ selective (compound **3**) as ring size increased. Since the differences in affinities are relatively small, however, none of these cyclic analogues exhibited a large preference for either κ or μ receptors. Cyclization of the peptides decreased binding affinity at δ receptors 2.5- to 4-fold, resulting in enhanced selectivity for κ or μ receptors over δ receptors. All of these cyclic peptides had similar affinity for δ receptors (K_i = 10 - 14 nM), indicating that ring size did not significantly affect affinity for these receptors.

Introduction of a D-amino acid at position 2 in the linear analogues of Dyn A-(1-13)NH_2 results in compounds that are either nonselective for κ vs. μ receptors (D-Asn^2) [Story et al., 1992] or are μ selective (e.g. D-Asp^2, **7**, and other D-amino acids [Story et al., 1992]). Introduction of the negatively charged D-Asp residue in position 2 reduced κ binding affinity 3-fold compared to the parent peptide, but enhanced affinity for μ receptors, an effect also seen for the D-Asn^2 analogue [Story et al., 1992]. Interestingly the anionic residue increased binding affinity for δ receptors almost 90-fold, an effect not seen with the D-Asn^2 analogue. The δ receptors preference for negative charges may explain this effect [Renugopalakrishnan et al., 1990].

Surprisingly, the Tmg linear byproducts exhibited high binding affinity for κ receptors, indicating that the bulky and positively charged Tmg group at position 5 did not adversely affect binding affinity to these receptors. This functional group did have a significant adverse effect on affinity for μ and δ receptors, however,

decreasing binding by 10- to 25-fold and 100- to 300-fold, respectively, compared to compound **7**. The longer length of the Tmg-containing side-chain in the fifth position reduced the δ binding affinity of compounds **5** and **6** by 2- and 3-fold, respectively, compared to compound **4**. This affect of chain length was not observed for κ or μ receptors. It should be noted that compounds **4 - 6** exhibited binding affinity for the three opioid receptor types similar to their corresponding cyclic analogues. This may be due to electrostatic interactions between the negative and positive charges of the D-aspartate and the Tmg functional groups, which could permit these linear peptides to adopt conformations similar to those of the cyclic peptides at opioid receptors.

To determine *in vitro* opioid activities, the peptides were tested for inhibition of electrically evoked contractions of the GPI (Table 6.3). All of the compounds inhibited muscle contractions in a dose-dependent manner with full agonist activity. Compounds **1**, **2** and **5** exhibited high potency with IC_{50}'s (0.16 - 0.23 nM) comparable to that of the parent peptide Dyn A-(1-13)NH_2. Compounds **3**, **4** and **7** showed good activity with potency 30-50% that of the parent peptide, whereas compound **6** was a weaker agonist (IC_{50} = 1.78 nM). Interestingly, the linear peptide without the negative charge [D-Asn^2]Dyn A-(1-13)NH_2 also exhibited weaker agonist activity (IC_{50} = 2.12 nM) [Story et al., 1992]. As observed in the binding assays, the ring size in the cyclic peptides had a slight affect on opioid activity, with opioid activity in the GPI decreasing slightly as the ring size increased. The potencies of the [D-Asp^2,Dap(Tmg)^5] and [D-Asp^2,Dab(Tmg)^5] byproducts were similar to that of [D-Asp^2]Dyn A-(1-13)NH_2, **7**. The length of the Tmg-containing side-chain in position 5

Table 6.3 Opioid Activity of cyclic Dyn A(1-13)NH_2 Analogues in the Guinea Pig Ileum

Compound No.	Analogues	IC_{50} (nM)[a]	Relative Potency (%)
1	cyclo[D-Asp^2,Dap^5]Dyn A(1-13)NH_2	0.16 (0.13-0.20)	138
2	cyclo[D-Asp^2,Dab5]Dyn A(1-13)NH_2	0.23 (0.18-0.31)	96
3	cyclo[D-Asp^2,Orn^5]Dyn A(1-13)NH_2	0.40 (0.30-0.54)	55
4	[D-Asp^2,Dap(Tmg)^5]Dyn A(1-13)NH_2	0.46 (0.36-0.58)	48
5	[D-Asp^2,Dab(Tmg)^5]Dyn A(1-13)NH_2	0.18 (0.14-0.23)	122
6	[D-Asp^2,Orn(Tmg)^5]Dyn A(1-13)NH_2	1.78 (0.92-3.46)	12
7	[D-Asp^2]Dyn A-(1-13)NH_2	0.46 (0.32- 0.67)	32
8	Dyn A(1-13)NH_2	0.22 (0.15-0.33)	100

[a] 95% confidence intervals are shown in parentheses.

affected the GPI activities; compound **5** was as potent as the parent peptide whereas compound **6** (longer length) and compound **4** (shorter length) were less potent (12 and 48% potency, respectively of Dyn A-(1-13)NH_2).

Whether the opioid activity of the cyclic peptides was mediated through κ or μ receptors was examined by evaluating naloxone sensitivity. The K_e values for naloxone antagonism of compounds **1**, **2**, **3** and Dyn A-(1-13)NH_2 were 64.9, 32.3, 17.1 and 64.0 nM, respectively. These results parallelled those found in the binding assays and indicated that compound **1** preferentially interacted with κ receptors in the GPI. The naloxone K_e for compound **3** indicated some κ selectivity for this compound in the GPI, a result which differs from that reported by Schiller et al. for the μ selective compound, cyclo[D-Orn^2,Asp^5]Dyn A(1-8)NH_2 (K_e = 1.49 nM) [Schiller et al., 1988]. The difference between these two compounds in opioid receptor selectivity in the GPI may be due to the truncation of the C-terminus in Schiller's peptide and/or reversal of the direction of the lactam bond. The 13-mer Dyn A-(1-13) has been reported to be a more κ selective ligand than the 8-mer Dyn A-(1-8) [Chavkin and Goldstein, 1981; Corbett et al., 1982], and the direction of a lactam bond may be involved in stabilizing or destabilizing helix formation [Shoemaker et al., 1987].

6.4.3 Circular Dichroism

Circular dichroism (CD), a sensitive method for evaluating peptide secondary structure, was used to examine possible structures of the cyclic peptides. In aqueous phosphate buffer (pH 7.4) at room temperature, all of the cyclic peptides and Dyn A-

(1-13)NH_2 exhibited CD spectra typical of a random structure (data not shown). TFE was therefore utilized to determine whether secondary structure could be induced in the constrained cyclic analogues or Dyn A-(1-13)NH_2. A comparison of the CD spectra of compound **1** and Dyn A-(1-13)NH_2, determined in 80%TFE at 5°C, is shown in Figure 6.4. Both peptides showed CD spectra indicative of helical structure, with minima at 222 and 205 nm and a large positive peak near 190 nm. The delta epsilon ($\Delta\varepsilon$) at 222 nm for compound **1** was somewhat greater than that observed for Dyn A-(1-13)NH_2, suggesting a slightly higher helical content in compound **1** compared to Dyn A-(1-13)NH_2 under these experimental conditions. The $\Delta\varepsilon$ or ellipticity ($[\theta]$, $[\theta] = 3300 \times \Delta\varepsilon$) at 222 nm has been used to calculate percent helical content of peptides, but the maximum ellipticity corresponding to 100% helicity may differ for different peptides [Bradley et al., 1990], which could introduce error into this calculation. Also the aromatic rings of Tyr and Phe can interfere with the ellipticity at 222 nm, resulting in an underestimate of the helical content in peptides [Chakrabartty et al., 1993]. Because of the limitations in evaluating secondary structure by CD, NMR studies are currently underway to examine the conformations of these cyclic analogues in more detail.

Cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2, **1**, is the constrained compound proposed to be compatible with an α-helix. While the helicity observed in the CD spectra for this cyclic peptide and for Dyn A-(1-13)NH_2 appears to be modest, it suggests that it is still possible for both compound **1** and Dyn A to adopt partial helical structures at κ receptors, as proposed by Schwyzer. NMR evidence supporting

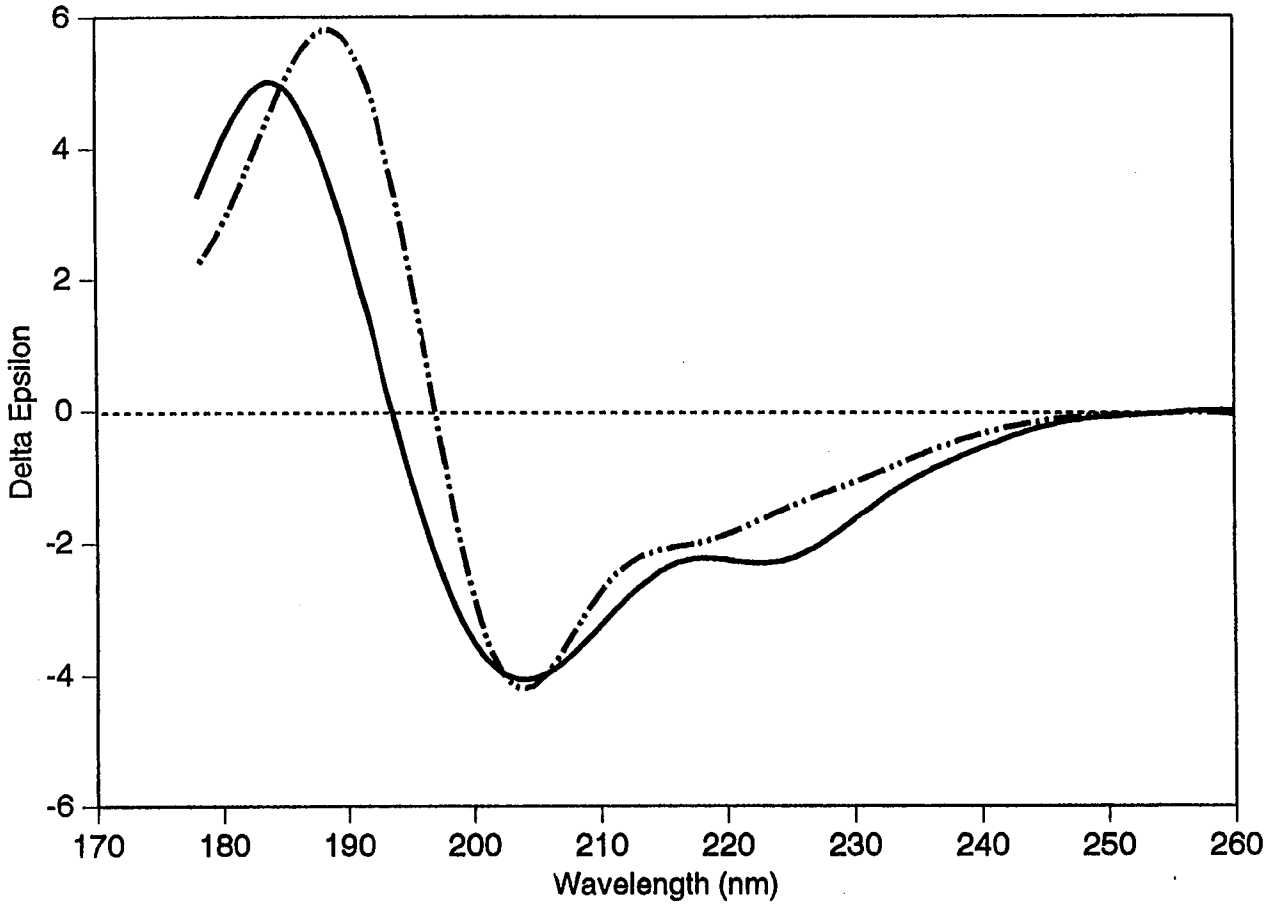


Figure 6.4 Circular dichroism spectra of cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2, 1(———) and Dyn A-(1-13)NH_2, 8 (··—··-) in 80% TFE at 5°C.

a partially helical structure for Dyn A interacting with membranes has recently been reporting. Based on transfer nuclear Overhauser effects plus molecular modeling, Epand and coworkers [1992] proposed a membrane-mediated conformation for Dyn A-(1-13) consisting of a short helical segment from Tyr^1 to Leu^5 plus a folded structure for residues Ile^8 to Pro^{10}. Recently Kallick [1993] has examined Dyn A-(1-17) bound to dodecylphosphocholine micelles by NMR and found that the peptide adopts a helix from Gly^3 to Arg^9. Thus, although there is still disagreement about the conformation Dyn A adopts at opioid receptors, a helical structure for part of this peptide is a promising candidate for the bioactive conformation of Dyn A at κ receptors. We are therefore continuing to examine constrained analogues of Dyn A with the potential to adopt a helical conformation.

6.5 Conclusions

Compounds **1-3** were synthesized in order to limit the comformational freedom of Dyn A-(1-13)NH_2. All of the analogues exhibited high opioid receptor affinities and potent opioid activity in the GPI. Among these cyclic analogues compound **1**, which contained a bridge length that molecular modeling suggested was the most compatible with an α helix, was a potent agonist, but exhibited only slight preference for κ over μ receptors in the radioligand binding assays. The high naloxone K_e value for this peptide in the GPI, however, indicated that this peptide preferentially interacted with κ receptors in this tissue, which is in contrast to the μ-selectivity found for cyclo[D-Orn^2,Asp^5]Dyn A-(1-18)NH_2, the only previously reported Dyn A analogue constrained via a lactam in the "message" region. The high potency in the

GPI and high affinity for κ receptors of compound **1** suggests that this peptide is capable of adopting the appropriate bioactive conformation for κ receptors found in the parent peptide. The constraint in cyclic peptide **1** still retains some flexibility and therefore could be compatible with other folded conformations (i.e. a β-turn), which could account for the peptide's affinity for other opioid receptors. Increasing the ring size to give compounds **2** and **3** tended to decrease selectivity and/or binding affinity at κ receptors. Interestingly, the novel Tmg byproducts, compounds **4-6**, also exhibited potent agonist activity in the GPI and high binding affinity for κ receptors, suggesting that they are also capable of adopting a bioactive conformation at opioid receptors and that the bulky tetramethylguanidinium group does not interfere with κ receptor interaction.

The CD spectra of both **1** and Dyn A-(1-13)NH_2 are both indicative of helical structure, with **1** exhibiting slightly greater helical content than Dyn A-(1-13)NH_2 in 80%TFE at 5°C. An extended conformation for the N-terminus of Dyn A-(1-13) has also been proposed based on fluorescent energy transfer of [Trp]^{4}Dyn A-(1-13) in aqueous solution [Schiller, 1983]. The affinity and activity of the cyclic peptides described here, particularly given the short length of the bridges in compounds **1** and **2**, would tend not to support such an extended conformation as a bioactive conformation for the N-terminus of Dyn A. These cyclic Dyn A analogues represent the first Dyn A analogue constrained in the "message" sequence with demonstrated high affinity for κ receptors and thus represent interesting lead compounds for further

characterization of structure- and conformation-activity relationships for Dyn A at κ opioid receptors. These studies are currently under way in our laboratory.

CHAPTER 7

SYNTHESIS AND OPIOID ACTIVITY OF CONFORMATIONALLY CONSTRAINED DYNORPHIN A ANALOGUES: PART II. CONFORMATIONAL CONSTRAINT IN THE "ADDRESS" SEQUENCE

Seksiri Arttamangkul[1], Jane Roth[1], Thomas F. Murray[1], David K. Grandy[3], Gary E. DeLander[1], Brigitte L. Kieffer[3], and Jane V. Aldrich[1,4]

A preliminary report of this work was presented at the International Narcotics Research Conference, North Falmouth, MA, July 16-21, 1994, *Regul. Pept.*, **1994**, *54*, 13-14.

---

[1] College of Pharmacy
[2] Vollum Institute for Advanced Biomedical Research and Department of Cell Biology and Anatomy, Oregon Health Sciences University, Portland
[3] Ecole Supérieure de Biotechnologie, Cedex, France
[4] Center for Gene Research and Biotechnology

Abbreviations:

Abbreviations used for amino acids follow the rules of the IUPAC-IUB Joint Commission of Biochemical Nomenclature in *Eur. J. Biochem.* **1984**, *138*, 9-37. Amino acids are in the L-configuration except when indicated otherwise. Additional abbreviations used are as follows: Boc, *tert*-butyloxycarbonyl; BOP, benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate; CHO, Chinese hamster ovary; ClZ, 2-chlorobenzyloxycarbonyl; Dab, α,γ-diaminobutyric acid; Dap, α,β-diaminopropionic acid; DAMGO, [D-Ala^2,MePhe^4,Gly-ol]enkephalin; DCM, dichloromethane; DIC, *N,N'*-diisopropylcarbodiimide; DIEA, *N,N*-diisopropyl-ethylamine; DMA, *N,N*-dimethylacetamide; DMF, *N,N*-dimethylformamide; DPDPE, cyclo[D-Pen^2,D-Pen^5]enkephalin; Dyn A, dynorphin A; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, (9-fluorenylmethoxy)carbonyl; GPB, guinea pig brain; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MBHA, 4-methylbenzhydrylamine; PAL® resin, Peptide Amide Linker or a methylbenzhydrylamine resin with a 5-(4-aminomethyl-3,5-dimethoxyphenoxy)valeric acid linker; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; PyBOP, benzotriazol-1-yl-oxy-tris-(pyrrolidino)phosphonium hexafluorophosphate; TFA, trifluoroacetic acid; Tos, 4-toluenesulfonyl.

7.1 Abstract

Several cyclic analogues of Dyn A-(1-13)NH_2 were prepared in order to reduce the flexibility in different regions of the native linear peptide. Cyclic[D-Asp^i, Dap^{i+3}]Dyn A-(1-13)NH_2 (Dap = α,β-diaminopropionic acid) analogues were designed based upon molecular modeling using AMBER, which suggested that this constraint may be compatible with an α-helix. The cyclic portion of these constrained analogues spanned from residues 3 to 9, a region proposed by Schwyzer to adopt a helical conformation at κ receptor sites. The analogues prepared in this study were cyclo[D-Asp^3,Dap^6]-, cyclo[D-Asp^5,Dap^8]- and cyclo[D-Asp^6,Dap^9]Dyn A-(1-13)NH_2. Analogues containing Dab (α,γ-diaminobutyric acid) or Orn in position *i+3* were also synthesized to examine the effects of larger ring size. The cyclic peptides exhibited marked differences in binding affinities for κ, μ and δ receptors and in opioid activity in the GPI. Cyclo[D-Asp^6,Dap^9]Dyn A-(1-13)NH_2 showed both high κ receptor affinity and potent agonist activity in the GPI, while cyclo[D-Asp^3,Dap^6]Dyn A-(1-13)NH_2 exhibited very weak binding affinity at all opioid receptors as well as very weak opioid activity in the GPI. Cyclo[D-Asp^5,Dap^8]Dyn A-(1-13)NH_2 showed moderate binding affinity for κ receptors and was the most κ selective ligand in this study, but this peptide exhibited very weak agonist activity in the GPI assay. Compared to the corresponding linear peptides all of the cyclic peptides exhibited decreased μ receptor affinity, while κ receptor affinity was retained or improved. Therefore the corresponding linear peptides were generally μ selective while the cyclic constrained peptides demonstrated slight selectivity for κ vs. μ receptors or

were non-selective. Increasing the ring size by incorporating Dab or Orn in positions 6, 8 or 9 did not significantly affect the binding affinity for the three opioid receptor types nor the opioid activity observed in the GPI.

7.2 Introduction

The study of the physiology and pharmacology of opioids has been complicated by the existence of multiple forms of both opioid receptors and their endogenous ligands [Simon and Hiller, 1994]. All of the mammalian endogenous opioid peptides are flexible molecules which can interact with more than one type of opioid receptor. The ability of these peptides to adopt numerous conformations may account for their significant affinity for different opioid receptors. One goal in opioid research is to develop ligands highly selective for each receptor type. These ligands can be both potential therapeutic agents and valuable pharmacological tools for understanding the biological effects produced by different opioid receptor types. Conformational constraint is one approach which can be used not only to restrict the flexibility of peptide molecules, but also to provide information on the topographical requirements of receptors [Hruby et al., 1990]. Highly potent and selective ligands may be found when appropriate conformational constraints are incorporated.

Dynorphin A (Dyn A) is a highly potent opioid peptide and postulated to be an endogeneous ligand for κ opioid receptors [Chavkin and Goldstein, 1982]. The selectivity of Dyn A for κ receptors, however, is low, with the peptide retaining significant affinity for μ and δ opioid receptors. Dyn A contains an identical N-terminal tetrapeptide sequence (the "message" sequence) as other mammalian opioid

peptides [Chavkin and Goldstein, 1981]. A number of studies [Chavkin and Goldstein, 1981; Yurcotte et al., 1984; Snyder et al., 1992; Story et al, 1992; Kawasaki et al., 1993b] have examined Dyn A fragments and amino acid replacement to determine the relative importance and structure-activity relationships for individual residues of Dyn A. It was found that the tetrapeptide sequence at the C-terminus (residues 14 to 17) of Dyn A is not necessary for opioid binding affinity and opioid activity [Goldstein et al., 1979]. Therefore Dyn A-(1-13) has been more frequently utilized as a prototype for further modification. Elimination of one amino acid at a time from the C-terminus suggested that the unusually high potency and κ receptor selectivity of Dyn A-(1-13) resulted from the C-terminal sequence (residues 6 to 13, the "address" sequence) [Chavkin and Goldstein, 1981]. An alanine scan of residues 1 through 11 [Turcotte et al., 1984] and substitutions of natural and unnatural amino acids at several positions [Snyder et al., 1992; Story et al., 1992; Kawasaki et al., 1993b] have supported the importance of the basic residues Arg^7 and Lys^{11} for both affinity and selectivity for κ receptors, and Arg^6 for κ receptor selectivity.

Similar to most linear peptides, Dyn A-(1-13) is capable of assuming a number of different conformations. In an aqueous environment, CD and 2D-NMR indicate that Dyn A-(1-13) does not have an ordered conformation [Renugopalakrishnan et al., 1990]. Conformationally constrained Dyn A analogues have been prepared in order to reduce this flexibility. The first conformationally constrained analogue of Dyn A-(1-13), prepared by Schiller and his colleages [Schiller et al., 1982], was the cyclic disulfide-containing analogue cyclized between $D\text{-}Cys^2$ and Cys^5. Although this cyclic

analogue is more potent than the linear parent peptide in the GPI assay, selective tolerance studies suggested that it does not interact well with κ receptors [Shearman et al., 1985]. Other cyclic disulfide-containing analogues of Dyn A have involved modifications in the "address" segment [Kawasaki et al., 1990; Kawasaki et al., 1993a; Meyer et al., 1994]. These cyclic analogues contained disulfide linkages between Cys, D-Cys, Pen (penicillamine) or D-Pen in positions 5 and 9, 10, 11, or 13; 6 and 10; and 8 and 12 or 13. Several of these cyclic Dyn A analogues exhibited high binding affinities for opioid receptors in guinea pig brain (GPB) membrane preparations, while their opioid activities in the guinea pig ileum (GPI) assay were very weak [Kawasaki et al., 1990; Kawasaki et al., 1993a; Meyer et al, 1994]. This significant difference in IC_{50}'s for the receptors in GPB vs. GPI suggested the possible existence of different subtypes of κ and μ receptors in the brain and peripheral systems. Cyclization via lactam bridges between D- or L-Orn and Asp have also been performed to give cyclo[D-Orn^2,Asp^5]Dyn A-(1-8)NH_2, and cyclo[Orn^5,Asp^8]-, cyclo[Orn^5,Asp^{10}]- and cyclo[Orn^5,Asp^{13}]Dyn A-(1-13)NH_2 [Schiller et al., 1988]. These analogues showed high affinity for μ receptors, however, and antagonism of their opioid activity in the GPI by low doses of naloxone suggested that these peptides did not interact significantly with κ receptors.

In his hypothesis of membrane compartmentalization for opioid receptors, Schwyzer has proposed that κ receptor sites are buried in the hydrophobic region of the lipid membrane bilayer [Schwyzer, 1986a]. Using equilibrium thermodynamic and kinetic estimations, he further examined the ability of the N-terminus "message"

sequence of Dyn A-(1-13) to insert into this hydrophobic environment and proposed that Dyn A-(1-13) adopts a helical conformation extending from Tyr^1 through Arg^9 [Schwyzer, 1986b]. We were interested in stabilizing this postulated helical structure of Dyn A through a lactam linkage. From our preliminary study of possible conformational constraints using the molecular modeling program AMBER [Weiner and Kollman, 1981; Weiner et al., 1984; Weiner and Kollman, 1986], we found that a four-atom bridge containing an amide bond between residues *i* (D-configuration) and *i+3* (L-configuration) appeared to be ideal for spanning the distance between C_i^{α} and C_{i+3}^{α} in an α-helix. We originally incorporated this constraint into the "message" sequence to give the analogue cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2 (Dap = α,β-diaminopropionic acid) containing this bridge between residues 2 and 5 (see chapter 6); the analogues with five- and six-atom bridges containing Dab (α,γ-diaminobutyric acid) and Orn, respectively, in position 5 were also synthesized in order to evaluate the effect on opioid activity and receptor affinity of varying the bridge length. All of the cyclic [2,5] Dyn A analogues were highly potent agonists in the GPI assay and had high affinity for κ receptors. Cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2 was slightly selective for κ over μ receptors, whereas increasing the ring size to give cyclo[D-Asp^2,Dab^5]- and cyclo[D-Asp^2,Orn^5]Dyn A-(1-13)NH_2 resulted in peptides which were nonselective for κ vs. μ receptors and slightly μ selective, respectively.

We further examined the effects of this constraint in different regions of Dyn A-(1-13)NH_2. The amino acid residues involved in the constraint were carefully selected such that they were not residues critical for κ receptor recognition. The

cyclic constraints spanned the sequence from residues 3 to 9, the region of Dyn A-(1-13) which has been proposed to be in an α-helix. The cyclic peptides synthesized were cyclo[D-Asp^3,Dap^6]-, cyclo[D-Asp^5,Dap^8]-, and cyclo[D-Asp^6,Dap^9]Dyn A(1-13)NH_2. Similar to the [2,5] cyclic series, the larger ring sizes with 5- and 6-atom bridges were also prepared to investigate the effect of bridge length. We evaluated the cyclic peptides and their corresponding linear peptides (Table 7.1) for affinity for cloned κ, μ and δ opioid receptors and for opioid activity in the GPI assay. The marked effect of these constraints on opioid receptor affinities and opioid activity is discussed in detail below.

7.3 Experimental

7.3.1 Materials

The reagents and instrumentation used were those described previously (see chapter 6) with the following modifications: the analytical HPLC column used was a C_4 Vydac analytical column (300Å, 5μ, 4.6 mm x 25 cm) equipped with a C_4 Vydac guard cartridge or a C_{18}Vydac analytical column with a C_{18} Vydac guard cartridge, and preparative reversed phase HPLC was performed on a Rainin gradient HPLC system using either a Dupont Zorbax Protein Plus preparative column (C_3, 300Å, 10μ, 21mm x 25cm) with a Dynamax (C_4, 12μ, 21 mm x 5 cm) guard cartridge or a C_{18} Vydac column (C_{18}, 300Å, 10-15 μ, 21 mm x 25 cm) with a C_{18} guard cartridge.

Table 7.1 Structure of cyclic and linear Dyn A-(1-13)NH_2 analogues

Compound	Dyn A-(1-13)NH_2 analogues
	[3,6] series
1	cyclo[D-Asp^3,Dap^6]Dyn A-(1-13)NH_2
2	cyclo[D-Asp^3,Dab^6]Dyn A-(1-13)NH_2
3	cyclo[D-Asp^3,Orn^6]Dyn A-(1-13)NH_2
4	[D-Asp^3,Dap^6]Dyn A-(1-13)NH2
5	[D-Asp^3]Dyn A-(1-13)NH_2
	[5,8] series
6	cyclo[D-Asp^5,Dap^8]Dyn A-(1-13)NH_2
7	cyclo[D-Asp^5,Dab^8]Dyn A-(1-13)NH_2
8	cyclo[D-Asp^5,Orn^8]Dyn A-(1-13)NH_2
9	[D-Asp^5,Dap^8]Dyn A-(1-13)NH_2
10	[D-Asp^5]Dyn A-(1-13)NH_2
	[6,9] series
11	cyclo[D-Asp^6,Dap^9]Dyn A-(1-13)NH_2
12	cyclo[D-Asp^6,Dab^9]Dyn A-(1-13)NH_2
13	cyclo[D-Asp^6,Orn^9]Dyn A-(1-13)NH_2
14	[D-Asp^6,Dap^9]Dyn A-(1-13)NH_2
15	[D-Asp^6]Dyn A-(1-13)NH_2
16	Dyn A-(1-13)NH_2

7.3.2 General method for solid-phase synthesis

Fmoc-amino acids were assembled on an MBHA resin (0.27 mmol/g resin substitution, 1% cross-linked polyvinyl styrene) as described elsewhere (see chapter 6), except that backbone couplings were performed with DIC (Aldrich) and HOBt (4 equiv of each). After the last amino acid was attached, the *t*-butyl side chain protecting groups were removed with 50% (v/v) TFA plus 2% anisole in DCM (5 min and then 30 min). The resin was washed extensively with DCM and MeOH and then neutralized with 10% DIEA in DCM prior to cyclization.

7.3.3 Cyclizations and purification of compounds 1-3 and 6-8

The cyclizations were performed on the peptide resins using BOP/DIEA (3/6 equiv) in DMF. Fresh coupling reagent (BOP/DIEA) was replaced daily until the reaction gave a negative or only a slightly blue response, which occured after 2-3 days. Then the *N*-terminal Fmoc protecting group was removed by 30% piperidine in DMA/toluene (1/1). The peptide was cleaved and all protecting groups were removed simultaneously by treatment with liquid HF plus 10% anisole at 0°C for 1 h. The crude peptides were desalted on a Sephadex G-10 column using 0.5 M AcOH as the eluent, and then further purified with preparative reverse phase HPLC using a gradient of 10 - 40% B in 45 min at a flow rate of 20 mL/min on a Protein Plus column (C_3) (solvent A was 0.1%TFA in water and solvent B was 0.1%TFA in AcCN). Pure fractions were then pooled and lyophilized, and the homogeneity of the peptides (> 98%) checked by analytical HPLC using a gradient of 0 - 75% B in 50

min at a flow rate of 1.5 mL/min on a 214TP54 (C_4) Vydac column (solvents A and B were the same as above).

7.3.4 Cyclizations and purification of compounds 11-13

The cyclizations were performed using fresh PyBOP/DIEA (3/6 equiv) in DMF which was replaced every 24 h. The reaction was terminated after 3-5 days when ninhydrin gave a negative or only a faint positive response. The peptides were deprotected and liberated from the solid support using the same procedure as described above. Crude peptides were desalted on a Sephadex G-10 column using 0.5 M AcOH and then purified on a 218TP1015 Vydac column (C_{18}) using a gradient of 20 - 50% B in 30 min at a flow rate of 20 mL/min (solvents A and B were the same as above). Additional purification of compounds **11** and **13** was done using isocratic preparative HPLC at 26% B and 20 mL/min to give pure peptides. The purity of peptides (> 98%) was determined on an analytical 218TP54 (C_{18}) Vydac column using the same gradient as described for compounds **1-3** and **6-8**.

7.3.5 Solid phase syntheses of linear peptides 4, 9 and 14

Protected peptides **4**, **9** and **14** were obtained from the same syntheses as compounds **1**, **6** and **11**, respectively. The *N*-terminal Fmoc protecting group was removed with 30% piperidine in DMA/toluene (1/1) after the last amino acid derivative was attached to the peptide-resin. The peptides were then cleaved and all side chain protecting groups were removed by liquid HF as described above. Crude

peptides were then extracted and purified by analogous procedures as described for their corresponding cyclic peptides.

7.3.6 Solid phase syntheses of linear peptides 5, 10 and 15

The linear peptides **5**, **10** and **15** were prepared on a PAL® resin (Peptide Amide Linker, Millipore, 0.31 mmol/g resin substitution) using standard Fmoc synthetic procedures [Snyder et al., 1992]. Following cleavage of the peptides with Reagent K (82.5% TFA, 5% water, 5% phenol, 5% thioanisole and 2.5% ethanedithiol) for 2 hours [King et al., 1990], the peptides were purified by reverse phase HPLC on a Protein Plus column (C_3) using a gradient of 0 - 50% AcCN containing 0.1% TFA in 50 min at a flow rate of 20 mL/min. The purity of the peptides (> 98%) was verified by analytical HPLC using a Vydac C_4 column as described above.

7.3.7 Receptor binding assays and smooth muscle assays

Opioid receptor binding studies were performed on membranes which expressed cloned κ, μ and δ receptors prepared from COS-7 cells [Kieffer et al., 1992] for compounds **1** - **4** or CHO (chinese hamster ovary) cells [Bunzow et al., 1995] for compounds **5** - **16**. Cells were harvested 72 h following transfection in 50 mM Tris buffer (pH 7.4 at 4°) and centrifuged at 45,000 x g for 10 min at 4°C. The pellet was washed twice and then resuspended in 50 mM Tris buffer to yield a protein concentration of 30 - 60 μg/mL. Incubations were performed for 90 min at 22°C with [^{3}H]diprenorphine, [^{3}H]DAMGO and [^{3}H]DPDPE for κ, μ and δ receptors,

respectively. Binding assays were carried out in the presence of peptidase inhibitors (10 μM bestatin, 30 μM captopril and 50 μM L-leucyl-L-leucine) and 3 mM Mg^{2+}. Nonspecific binding was determined in the presence of 10 μM unlabeled Dyn A-(1-13)NH_2, DAMGO and DPDPE for κ, μ and δ receptors, respectively. The reactions were terminated by rapid filtration over Whatman GF/B fiber filters using a Brandel M24-R Cell Harvester. The filters had been presoaked for 2 h in 0.5% polyethyleneimine to decrease nonspecific binding. The filter disks were then placed in minivials with 4 mL Cytocint (ICN Radiochemicals) and allowed to elute for 6 h before counting in a Beckman LS6800 scintillation counter. The IC_{50} values derived from competition analyses were converted to dissociation constants (K_i) using the Cheng and Prusoff equation [Cheng and Prusoff, 1973]; K_D's for [^{3}H]diprenorphine, [^{3}H]DAMGO and [^{3}H]DPDPE were 0.45, 0.49 and 1.76 nM, respectively.

The GPI assays were performed as described in detail elsewhere [Story et al.,1992], except that peptidase inhibitors (10 μM bestatin and 0.3 μM thiorphan) were added to the tissue bath 5 min before each experiment. IC_{50} values reported were obtained from at least 3 replicates in tissues from different animals. The pA_2 values were also determined from at least 3 replicates in different animals using Schild plots [Arunlakshana and Schild, 1959] at four different doses of naloxone (10, 100, 300 and 1000 nM).

7.4 Results and Discussion

7.4.1 Chemistry

All of the cyclic peptides were synthesized on an MBHA resin by procedures similar to those described previously (see chapter 6). After incorporation of the last amino acid to the peptide chains, the *t*-butyl protecting groups were removed with TFA and the cyclizations then performed on the resin using either BOP (BOP/DIEA, 3/6 equiv, compounds **1** - **3** and **6** - **8**) or PyBOP (PyBOP/DIEA, 3/6 equiv, compounds **11** - **13**). Cyclization reactions were monitored by ninhydrin, and gave negative or slightly positive responses within 2 - 5 days. After the cyclizations the N-terminal Fmoc protecting groups were removed and the peptide amides then liberated from the resins using liquid HF. In all cases, the desired cyclic peptides were the major components (50 - 60% by reverse phase HPLC). The identities of the cyclic peptides were verified by FAB-MS and amino acid analysis following purification using gel filtration and reverse phase HPLC (Table 7.2). The slow cyclization reactions observed in this study were consistent with the results for cyclization of [D-Asp^2,Dap^5]Dyn A-(1-13)NH_2, in which the cyclization using BOP required 3 days to give a nearly complete reaction (see chapter 4). These difficult cyclizations may be due to the tight constraint of the ring portion in which the side chains involved in the cyclization are separated by only two amino acid residues. The different positions and sizes of the ring portion in these cyclic Dyn A analogues did not exhibit any large affect on rate of the cyclization or the purity of the resulting products.

Table 7.2 Analytical Data for Dyn A-(1-13)NH_2 Analogues

Analogues	FAB-MS	Amino Acid Compositions[a]									
	(M+1)	Tyr	Gly	Phe	Leu	Arg	Ile	Pro	Lys	Asp	X
1	1574	0.98 (1)	1.00 (1)	0.99 (1)	2.02 (2)	1.99 (2)	0.94 (1)	1.00 (1)	2.12 (2)	0.99 (1)	0.98[b] (1)
2	1587	0.96 (1)	0.99 (1)	1.01 (1)	2.04 (2)	1.96 (2)	1.00 (1)	0.98 (1)	2.07 (2)	0.98 (1)	1.00[c] (1)
3	1601	0.99 (1)	0.99 (1)	1.01 (1)	2.02 (2)	2.04 (2)	1.01 (1)	1.02 (1)	2.05 (2)	0.96 (1)	0.90[d] (1)
4	1592	0.98 (1)	1.01 (1)	0.99 (1)	2.03 (2)	1.99 (2)	0.94 (1)	0.94 (1)	2.13 (2)	0.99 (1)	0.99[b] (1)
5	1661	0.99 (1)	0.99 (1)	1.02 (1)	2.03 (2)	2.98 (3)	0.86 (1)	1.03 (1)	2.07 (2)	0.99 (1)	-
6	1560	0.98 (1)	2.00 (2)	1.01 (1)	1.03 (1)	3.05 (3)	-	0.98 (1)	1.94 (2)	1.00 (1)	1.45[b] (1)
7	1574	0.98 (1)	1.97 (2)	0.99 (1)	1.05 (1)	2.99 (3)	-	1.00 (1)	2.05 (2)	0.97 (1)	1.07[c] (1)
8	1587	0.99 (1)	2.00 (2)	1.01 (1)	1.05 (1)	3.05 (3)	-	1.00 (1)	1.97 (2)	0.95 (1)	1.04[d] (1)
9	1577	1.01 (1)	1.93 (2)	1.01 (1)	1.00 (1)	2.96 (3)	-	1.08 (1)	1.97 (2)	1.03 (1)	1.30[b] (1)
10	1605	0.99 (1)	1.96 (2)	0.98 (1)	1.04 (1)	2.96 (3)	0.96 (1)	1.03 (1)	2.03 (2)	1.00 (1)	-
11	1474	0.98(1)	2.00 (2)	1.04 (1)	2.06 (2)	1.05 (1)	0.85 (1)	1.02 (1)	2.09 (2)	1.03 (1)	0.89[b] (1)
12	1488	0.98 (1)	2.00 (2)	1.01 (1)	2.00 (2)	1.03 (1)	1.02 (1)	0.95 (1)	2.07 (2)	0.98 (1)	0.95[c] (1)
13	1502	1.00 (1)	1.97 (2)	1.01 (1)	2.00 (2)	1.04 (1)	1.04 (1)	0.95 (1)	2.06 (2)	0.99 (1)	0.96[d] (1)
14	1492	0.99 (1)	2.00 (2)	0.99 (1)	2.03 (2)	1.04 (1)	0.92 (1)	1.02 (1)	2.06 (2)	1.01 (1)	0.94[b] (1)
15	1561	1.00 (1)	1.96 (2)	0.97 (1)	2.16 (2)	1.97 (2)	1.00 (1)	0.97 (1)	2.03 (2)	1.00 (1)	-

[a] Theoretical values are shown in parentheses.
[b] Dap
[c] Dab
[d] Orn

The linear peptides **4**, **9** and **14** were obtained from the same syntheses as their corresponding cyclic peptides, except that the cyclization steps were omitted. Compounds **5**, **10** and **15** were synthesized on a PAL® resin using a standard Fmoc synthetic protocol. In this case, side chains of the amino acids were protected with TFA-labile functional groups. The side-chain protecting groups were removed simultaneously when the peptides were liberated from the resin using Reagent K [King et al., 1990]. Generally, the peptides were obtained in very good yields and all of them exhibited the expected results by FAB-MS and amino acid analysis (Table 7.2). In the case of compound **15**, reverse phase HPLC showed two distinct peptide products which after purification were characterized using FAB-MS. The major component (75%) was the desired peptide **15**, while FAB-MS suggested that the minor component (25%) was the peptide containing one residual Pmc protecting group.

7.4.2 Receptor affinities and opioid activity

The peptides were evaluated for their binding affinity to κ, μ and δ opioid receptors using competition binding assays. The opioid binding affinities of compounds **1** - **4** were determined using cloned receptors transiently expressed on COS-7 (monkey kidney) cells, and the affinities of compounds **5** - **16** were evaluated using cloned receptors stably expressed on CHO cells. The affinities for κ, μ and δ receptors were determined by competitive binding against the radioligands [^{3}H]diprenorphine, [^{3}H]DAMGO and [^{3}H]DPDPE, respectively.

The opioid binding affinities of the compounds for κ, μ and δ receptors are summarized in Table 7.3. Cyclic Dyn A-(1-13)NH_2 analogues constrained in different regions exhibited markedly different binding affinities for the three opioid receptor types. The cyclic [6,9] Dyn A analogues (**11** - **13**) exhibited high binding affinity for κ and μ receptors (K_i = 1.5 - 4.8 nM). The binding affinities at these two receptor sites decreased when the cyclic contraint bridged residues 5 and 8 (K_i = 8.0 - 75 nM) and residues 3 and 6 (K_i = 200 - 910 nM). Increasing ring size in each series generally did not cause large changes in the binding affinities for κ and μ receptors; binding affinities varied by < 2-fold, except for the cyclic [3,6] analogues where the peptides with the longer bridge lengths (**2** and **3**) exhibited 4- and 3-fold higher affinities, respectively, than **1** for μ receptors. At δ receptors, the binding affinities of the cyclic [3,6] and [5,8] peptides were very weak (generally > 1 μM), whereas the δ receptor affinities of the cyclic [6,9] peptides were comparable to or only slightly lower than that of the parent peptide Dyn A-(1-13)NH_2, **16**. In each series the binding affinities for κ and μ receptors of the cyclic peptides were reversed compared to the corresponding linear peptides; the only exception to this was the linear peptide **4** which exhibited very low affinity at κ receptors similar to that of cyclic peptide **1**. These cyclic peptides tended to have increased affinity for κ receptors, but decreased affinity at μ receptors when compared to the related linear peptides.

Table 7.3 Opioid receptor binding affinities of Dyn A-(1-13)NH_2 analogues

	Dyn A-(1-13)NH_2 analogues	K_i (nM)			
		κ	μ	δ	K_i ratio ($\kappa/\mu/\delta$)
	[3,6] series				
1	cyclo[D-Asp^3,Dap^6]-	480 (410-580)	910 (470-1800)	2800 (1400-5400)	1/1.9/5.8
2	cyclo[D-Asp^3,Dab^6]-	270 (220-330)	210 (150-290)	1000 (610-1600)	1.3/1/4.8
3	cyclo[D-Asp^3,Orn^6]-	200 (160-270)	320 (250-410)	320 (140-740)	1/1.6/1.6
4	[D-Asp^3,Dap^6]-	300 (240-390)	100 (59-180)	1300 (710-2500)	3/1/13
5	[D-Asp^3]-	28 (18-36)	180 (120-280)	430 (320-460)	1/6.4/15
	[5,8] series				
6	cyclo[D-Asp^5,Dap^8]-	8.0 (6.2-10)	75 (61-93)	3300 (2400-4400)	1/9.4/410
7	cyclo[D-Asp^5,Dab^8]-	14 (11-18)	40 (32-50)	4100 (3200-5100)	1/2.9/290
8	cyclo[D-Asp^5,Orn^8]-	12 (11-14)	62 (50-76)	1900 (1300-2800)	1/5.2/160
9	[D-Asp^5,Dap^8]-	20 (14-29)	8.3 (5.9-12)	1600 (1400-2000)	2.4/1/190
10	[D-Asp^5]-	58 (40-86)	6.1 (5.2-7.2)	1300 (1100-1600)	9.5/1/210

[a] 95% confidence intervals are shown in parentheses.

Table 7.3 Opioid receptor binding affinities of Dyn A-(1-13)NH_2 analogues (continued)

	Dyn A-(1-13)NH_2 analogues	K_i (nM)			
		κ	μ	δ	K_i ratio $\kappa/\mu/\delta$
	[6,9] series				
11	cyclo[D-Asp^6,Dap^9]-	2.6 (2.0-3.4)	4.4 (3.0-6.5)	48 (27-68)	1/1.7/19
12	cyclo[D-Asp^6,Dab^9]-	2.5 (1.9-3.3)	4.8 (3.7-6.1)	180 (170-180)	1/1.9/72
13	cyclo[D-Asp^6,Orn^9]-	1.5 (1.2-1.9)	3.2 (2.3-4.3)	28 (25-29)	1/2.1/19
14	[D-Asp^6,Dap^9]-	12 (8.7-15)	1.5 (1.0-2.5)	8.2 (7.7-8.6)	8/1/5.5
15	[D-Asp^6]-	8.0 (6.4-9.9)	1.2 (0.8-1.7)	22 (18-25)	6.7/1/18
16	Dyn A-(1-13)NH_2	0.31 (0.14-0.69)	0.92 (0.57-1.5)	28.4	1/3/92

[a] 95% confidence intervals are shown in parentheses.

Among the cyclic Dyn A analogues examined in this study, only the cyclic [6,9] analogues, **11** - **13**, exhibited high binding affinity for κ opioid receptors (K_i = 1.5 - 2.6 nM). These cyclic peptides also had considerable affinity for μ opioid receptors (K_i = 3.2 - 4.4 nM), and therefore exhibited negligible selectivity for κ vs. μ receptors. The related linear peptide [D-Asp^6,Dap^9]Dyn A-(1-13)NH_2, **14**, exhibited a 5-fold decrease in binding affinity for κ receptors, but a 3-fold increase in affinity for μ receptors compared to the cyclic peptide **11**. Thus the linear peptide **14** was a moderately μ-selective ligand, whereas the cyclic peptide **11** did not distinguish between κ and μ receptors. Introduction of a residue with the D-configuration and/or a negative charge at position 6 may be responsible for the decreased κ receptor affinity of **14**. Compound **15**, which also contains a D-Asp at position 6, showed a similar binding profile to **14** at κ and μ receptors and bound preferentially to μ over κ receptors. Compared to Dyn A-(1-13)NH_2, **16**, both compounds **14** and **15**, however, exhibited 40- and 26-fold decreases in κ receptor affinity while retaining similar K_i's for μ receptors. While it was previously observed that a positive charge on residue 6 was not critical for κ receptor recognition [Snyder et al., 1992], it is not surprising that a negative charge at this position could adversely affect the binding at κ receptor sites.

The cyclic peptides in the [5,8] series, **6** - **8**, exhibited modest binding affinity for κ receptors (K_i = 8.0 - 12 nM), but their weaker affinity for μ receptors (K_i = 40 - 75 nM) resulted in peptides with selectivity for κ over μ receptors comparable to or slightly greater than that of Dyn A-(1-13)NH_2. The cyclic peptide **6** was the most κ

selective ligand in this series and exhibited 3-fold higher selectivity for κ over μ receptors than Dyn A-(1-13)NH_2. In contrast to the cyclic peptide **6**, the corresponding linear peptide **9** exhibited a 2.5-fold decrease in binding affinity for κ receptors but a 9-fold increase in binding affinity for μ receptors compared to **6**, resulting in a compound with a slight selectivity for μ receptors. The decrease in κ receptor affinity for compound **9** may be the result of both the D-configuration and the negative charge at position 5, since compound **10** also exhibited weak binding affinity for κ receptors. The negative charge on the aspartate residue probably contributed to the adverse effect on κ receptor affinity, since compound **10** had a 190-fold decrease in binding affinity for κ receptors compared to Dyn A-(1-13)NH_2, while inversion of the stereochemistry at position 5 of Dyn A-(1-11)NH_2 to give [D-Leu]^{5}Dyn A-(1-11)NH_2 (IC_{50}^{κ} = 15.3 nM) resulted in a 26-fold decrease in κ receptor affinity compared to Dyn A-(1-11)NH_2 [Meyer et al., 1994]. It is interesting to note that for the linear peptides a D-Asp at position 5 caused a larger decrease in κ receptor affinity than a D-Asp at position 6; K_i's for compounds **10** and **15** were 58 and 8.0 nM, respectively. This charged group in position 5 of compound **10** also decreased the binding affinities at μ and δ receptors 7- and 46-fold, respectively, compared to the parent peptide **16**; negligible effects were observed in the affinities at μ and δ receptors for the negatively charged group in position 6 of **15**. The large decrease in δ receptor affinity for **10** was probably due to the D-configuration at position 5 since [D-Leu^5]Dyn A(1-11)NH_2 also possesses very weak binding affinity for δ receptors (IC_{50} = 1740 nM) [Meyer et al., 1994]. All of the cyclic analogues

6 - **8** containing a D-amino acid in position 5 also exhibited a large decrease (68- to 150-fold) in δ receptor affinity.

The cyclic [3,6] Dyn A analogues exhibited large decreases in binding affinities for all three opioid receptor types. The linear peptide **4** containing D-Asp^3 and Dap^6 also exhibited very weak binding affinities at κ, μ and δ opioid receptors. The binding affinities at κ receptors of the cyclic constraint **1** and its corresponding linear peptide **4** were very similar. The 9-fold decrease in μ receptor affinity of **1** compared to **4** therefore resulted in **1** exhibiting a slight preference for binding at κ over μ receptors, while **4** exhibited a slight preference for μ receptors.

A D-Asp at position 3 in compound **5** decreased binding affinities for κ and μ receptors 100- and 200-fold, respectively, as compared to the parent peptide, **16**. In contrast, the binding affinity for κ receptors of [D-Ala^3]Dyn A-(1-11)NH_2 did not change compared to Dyn A-(1-11)NH_2 while the binding affinity for μ receptors was markedly decreased, resulting in a highly κ selective ligand [Lung et al., 1994]. Therefore the decrease in κ receptor affinity of compound **5** may be related to the negative charge on residue 3.

A large decrease in κ receptor affinity was unexpectedly observed for compound **4** when Arg^6 of **5** was replaced with Dap^6. Since at physiological pH the side-chain of the Dap residue (pK_a = 9.6) [Hay et al., 1971] could have a positive charge similar to Arg, the difference may be related to the length of the methylene chain holding this charged group. Schwyzer proposed that the methylene chains of Arg^6, Arg^7 and Arg^9 function as "snorkels" holding the positive charges to the

membrane surface [Schwyzer, 1992]. The methylene chain of the side-chain Dap, however, may not be long enough to adopt this "snorkel" structure.

The opioid activity of the Dyn A analogues was determined in the GPI assay (Table 7.4). For cyclic [6,9] peptides and their linear analogues the results correlated well with the binding assays; these compounds all had high affinity for both κ and μ receptors, and exhibited reasonable agonist potency in the GPI (IC_{50} = 7 - 46 nM). Interestingly, the ring size in the cyclic [6,9] analogues showed a larger affect on opioid activity in the GPI assays (7-fold difference in IC_{50} values) than in the binding assays (< 2 fold difference in K_i values); increasing the ring size increased the agonist potency in the GPI. The pA_2 values (95% confidence interval) for naloxone antagonism of componds **11**, **12** and **13** were 7.77 (7.45 - 8.08), 7.62 (7.45 - 7.79) and 7.87 (7.76 - 7.98), respectively. These values are comparable to the pA_2 values of 7.65 (7.47 - 7.83) for Dyn A-(1-13)NH_2 suggesting that compounds **11** - **13** produced their opioid activity in the GPI predominantly by interacting with κ receptors. For the linear peptides **14** and **15**, the pA_2 values for antagonism by naloxone were 7.88 (7.74 - 8.02) and 8.07 (7.92 - 8.22), respectively. These pA_2 values are intermediate between those of Dyn A-(1-13)NH_2 and morphine (pA_2 = 8.2 (7.9 - 8.5)) [Choi et al., 1992], and therefore suggest that both κ and μ receptors were responsible for the opioid activity of these compounds in the GPI.

Table 7.4 Opioid activity of cyclic Dyn A analogues in the GPI

	Dyn A-(1-13)NH_2 analogues	IC_{50}(nM)[a]
	[3,6] series	
1	cyclo[D-Asp^3,Dap^6]-	1000 (540-2200)
2	cyclo[D-Asp^3,Dab^6]-	880 (460-1800)
3	cyclo[D-Asp^3,Orn^6]-	2700 (860-9100)
4	[D-Asp^3,Dap^6]-	2800 (1600-4900)
5	[D-Asp^3]-	440 (270-720)
	[5,8] series	
6	cyclo[D-Asp^5,Dap^8]-	>5000
7	cyclo[D-Asp^5,Dab^8]-	1300 (980-1700)
8	cyclo[D-Asp^5,Orn^8]-	530 (420-680)
9	[D-Asp^5,Dap^8]-	640 (300-1400)
10	[D-Asp^5]-	1400 (520-4000)
	[6,9] series	
11	cyclo[D-Asp^6,Dap^9]-	46 (36-60)
12	cyclo[D-Asp^6,Dab^9]-	22 (13-35)
13	cyclo[D-Asp^6,Orn^9]-	6.8 (4.4-11)
14	[D-Asp^6,Dap^9]-	74 (31-180)
15	[D-Asp^6]-	29 (20-42)
16	Dyn A-(1-13)NH_2	0.19 (0.13-0.26)

[a] 95% confidence intervals are shown in parentheses.

The cyclic [3,6] Dyn A analogues and their related linear peptides were all very weak agonists in the GPI assay (IC_{50} = 0.9 - 2.7 μM; Table 7.4), results which also correlated well with those observed in the binding assays.

The cyclic [5,8] Dyn A-(1-13)NH_2 analogues were very weak agonists (IC_{50} = 0.5 - 5 μM) in the GPI, a result which did not correlate well with their reasonable binding affinity for κ receptors (K_i = 8 - 12 nM; Table 7.3). Although compound **6** exhibited the weakest agonist activity in the GPI, this peptide at a concentration of 1 μM did not show any antagonism against Dyn A-(1-13)NH_2 or morphine. Compound **6** was the most κ selective ligand in this series and also exhibited the most significant difference between binding and opioid activity, with a ratio of > 600 for the IC_{50} in the GPI vs. K_i at κ receptors (see Table 7.5). The μ-selective peptide **10** showed a ratio of 200 for the IC_{50} in the GPI vs. K_i at μ receptors. Increasing the ring size in the cyclic [5,8] analogues increased potency in the GPI, which resulted in a decrease in the ratio between the opioid activity in the GPI and the binding affinity for κ receptors for compounds **7** and **8**. Schiller's μ-selective analogue cyclo[Orn^5,Asp^8]-Dyn A-(1-13)NH_2 also exhibited this phenomenon, with a ratio of 290 for the IC_{50} in the GPI and K_i at μ receptors. Hruby and his coworkers reported similar significant differences for binding affinity in guinea pig brain (GPB) and opioid activity in the GPI for some of their cyclic disulfide analogues involving a constraint in the "address" region, particularly when the residue in position 5 was involved in the constraint [Kawasaki et al., 1990; Kawasaki et al, 1993a; Meyer et al., 1994]. These

Table 7.5 The ratio of potency in the GPI assay to binding affinity for opioid receptors

	Dyn A-(1-13)NH_2	GPI IC_{50}/K_i ratios[a]
	[3,6] series	
1	cyclo[D-Asp^3,Dap^6]-	2
2	cyclo[D-Asp^3,Dab^6]-	3
3	cyclo[D-Asp^3,Orn^6]-	10
4	[D-Asp^3,Dap^6]-	30[b]
5	[D-Asp^3]-	16
	[5,8] series	
6	cyclo[D-Asp^5,Dap^8]-	> 600
7	cyclo[D-Asp^5,Dab^8]-	90
8	cyclo[D-Asp^5,Orn^8]-	40
9	[D-Asp^5,Dap^8]-	80[b]
10	[D-Asp^5]-	200[b]
	[6,9] series	
11	cyclo[D-Asp^6,Dap^9]-	20
12	cyclo[D-Asp^6,Dab^9]-	10
13	cyclo[D-Asp^6,Orn^9]-	5
14	[D-Asp^6,Dap^9]-	50[b]
15	[D-Asp^6]-	20[b]
16	Dyn A-(1-13)NH_2	0.6

[a] The K_i used are for binding to κ receptors was used except where otherwise noted.

[b] K_i values for binding to μ receptors were used in the calculations since the compound had higher affinity for μ than κ receptors.

results were postulated to be due to the existence of different receptor subtypes for κ or μ opioid receptors in central and peripheral nervous systems.

7.5 Conclusions

Cyclic constraints incorporated in different regions of Dyn A-(1-13)NH_2 resulted in differences in binding profiles for κ and μ receptors and in opioid activity in the GPI assay. The cyclic [D-Asp^i,Dap^{i+3}] constraint needs to be incorporated into appropriate positions to retain high binding affinity for κ receptors and high potency in the GPI. Cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2 gave the best results, with a K_i for κ receptors and an IC_{50} in the GPI of less than 1 nM (see chapter 6), whereas cyclo[D-Asp^3,Dap^6]Dyn A-(1-13)NH_2 exhibited the lowest binding affinity and potency in the GPI. Constraints in the "address" region between positions 5 and 8, or 6 and 9 also provided useful information for further analogue design, since these peptides still bound to κ receptors with modest to high affinity.

Cyclo[D-Asp^5,Dap^8]Dyn A-(1-13)NH_2 was an interesting compound because the binding affinity for cloned receptors (from rat brain) did not correlate well with opioid activity in the GPI. In contrast, cyclo[D-Asp^6,Dap^9]Dyn A-(1-13)NH_2 exhibited both high κ receptor affinity and modest opioid activity in the GPI, although it was not as potent in either assay as cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2 (see chapter 6). Increasing ring size in the [5,8] and [6,9] series increased potency in the GPI, while the binding affinities for κ and μ receptors did not change substantially. Incorporation of D-Asp alone was not well tolerated at any of the positions and all of

the linear peptides containing D-Asp had lower binding affinity for κ receptors and weaker opioid activity in the GPI compared to the parent peptide, **16**.

All of the cyclic analogues containing a 4-atom bridge exhibited some selectivity for κ over μ receptors, although K_i ratios were generally small. Compared to their corresponding linear peptides which were μ-selective, the constraint of the lactam between D-Asp^i and Dap^{i+3} adversely affected μ receptor affinity while either improving or not affecting binding affinity at κ receptors. For the peptides containing a larger ring size with a 5- or 6-atom bridge, their selectivity for κ and μ receptors varied depending upon the positions involved. These results support the hypothesis of this study that the cyclization between D-Asp^i and Dap^{i+3} could be used to increase κ receptor selectivity.

The cyclic [D-Asp^i,Dap^{i+3}]Dyn A analogues synthesized in our group provides some information about the conformation Dyn A adopts at κ receptors. The small ring size in cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2, a peptide which possesses high κ receptor affinity and potent agonist activity in the GPI, suggests that Dyn A-(1-13)NH_2 tends to form a folded structure in the "message" sequence (see chapter 6). A folded structure may occur in the "address" sequence as well, since cyclo[D-Asp^6,Dap^9]Dyn A-(1-13)NH_2 exhibited high binding affinity at κ receptors and strong opioid activity in the GPI. These results suggest that Dyn A may fold into a compact structure at κ receptors.

CHAPTER 8
SUMMARY AND FUTURE STUDIES

The primary objective of this study was to examine whether an α-helix might be a biologically active conformation of Dyn A-(1-13) when it binds to κ opioid receptors. The constraint cyclo[D-Asp^{i},Dap^{i+3}]Dyn A-(1-13)NH_2 was designed to be compatible with this helical structure. Four series of cyclo[D-Asp^{i},Dap^{i+3}]Dyn A-(1-13)NH_2 analogues were synthesized and evaluated for their opioid binding affinities and opioid activities. Cyclic analogues containing Dab and Orn at position *i+3* were also prepared to examine the effect of a larger ring size. Several related linear Dyn A analogues were synthesized to examine the effects of the individual residues that were involved in the lactam formation.

The cyclic Dyn A analogues in this study exhibited marked differences in opioid binding affinities and opioid activities depending upon the region where the cyclic constraint was incorporated. Of all these analogues, cyclic [2,5] Dyn A-(1-13)NH_2 analogues exhibited the highest binding affinity at κ receptors and the highest opioid activity in the GPI, while cyclic [3,6] Dyn A-(1-13)NH_2 analogues had the weakest κ receptor affinity and opioid activity. Cyclic [6,9] Dyn A-(1-13)NH_2 analogues also exhibited high κ receptor affinity and high opioid activity in the GPI. For cyclic [5,8] Dyn A-(1-13)NH_2 analogues, the opioid binding affinities examined from cloned receptors were found not to correlate well with their opioid activities in the GPI; they had moderate κ binding affinity but weak opioid activity in the GPI. Among these cyclic [5,8] analogues, cyclo[D-Asp^{5},Dap^{8}]Dyn A-(1-13)NH_2 exhibited

the greatest difference between the K_i for binding to κ receptors and the IC_{50} for opioid activity in the GPI. This observation was postulated to be due to the existence of different receptor subtypes in central and peripheral nervous systems [Kawasaki et al., 1990; Kawasaki et al., 1993a; Meyer et al., 1994].

Cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2, an analogue which contained the constraint postulated to be compatible with an α-helix, exhibited high binding affinity for κ receptors and high opioid activity in the GPI similar to those of Dyn A-(1-13)NH_2. Conformational analysis using CD of cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2 in 80% TFE at 5°C revealed that this peptide could adopt an α-helix to some extent. In addition, the helical content of this cyclic analogue was slightly higher than that of Dyn A-(1-13)NH_2 under these conditions.

There are some speculations concerning the differences in opioid activity and opioid binding affinity observed for these cyclic Dyn A analogues. These speculations involve the compatibility of the constraint with an α-helix, the location of the lactam bridge in the helix wheel and the substitution of particular amino acid residues in Dyn A.

The constraint designed in this study still possesses some flexibility and may adopt other turn structures, i.e. a β-turn. This constraint when incorporated between positions 2 and 5 of Dyn A-(1-13)NH_2 may not affect the initiation of helical structure of cyclo[D-Asp^2,Dap^5]Dyn A-(1-13)NH_2, and therefore the helix could propagate through the remaining amino acid residues (residues 6 through 9). Similar propagation may occur when the constraint is incorporated between positions 5 and 8,

or 6 and 9. When the constraint is incorporated between positions 3 and 6, the constraint may not be compatible with helix propagation since the number of residues outside the constrained ring of cyclic [3,6] analogues are not sufficient to form even one turn of an α-helix (see Figure 8.1).

If the N-terminus of the peptide adopts a helical structure, then when the constraint is incorporated between positions 2 and 5 or 6 and 9, the lactam bridges of these two constraints would be located on the opposite side in the helix wheel from residues Tyr^1, Phe^4 and Arg^7 (see Figure 8.2) which are critical for κ receptor recognition and transduction. The constraint in these two cyclic series was compatible with high κ receptor affinity and opioid activity for the cyclic [2,5] and [6,9] Dyn A analogues. For the cyclic [5,8] analogues, the lactam bridge is located on the same side of the helix wheel as Tyr^1 (Figure 8.2). Although this lactam bridge is one turn below Tyr^1, the dipole characteristic of an amide in this region may affect the interaction of these cyclic [5,8] analogues with κ receptor sites. Therefore, cyclic [5,8] analogues exhibited only moderate binding affinity at κ receptors.

In the case of the cyclic [3,6] analogues, the lactam bridge is also on the opposite side of the critical residues in the helix wheel (Figure 8.2). Since the linear peptides [D-Asp^3,Dap^6]-, [D-Asp^6]- and [D-Asp^6,Dap^9]Dyn A-(1-13)NH_2 also exhibited large decreases in both κ receptor affinity and opioid activity in the GPI compared to Dyn A-(1-13)NH_2, therefore, the lactam bridge may not be related to the weak κ receptor affinity and opioid activity of these cyclic [3,6] analogues, but instead the substitution at position 6 may be responsible for this effect.

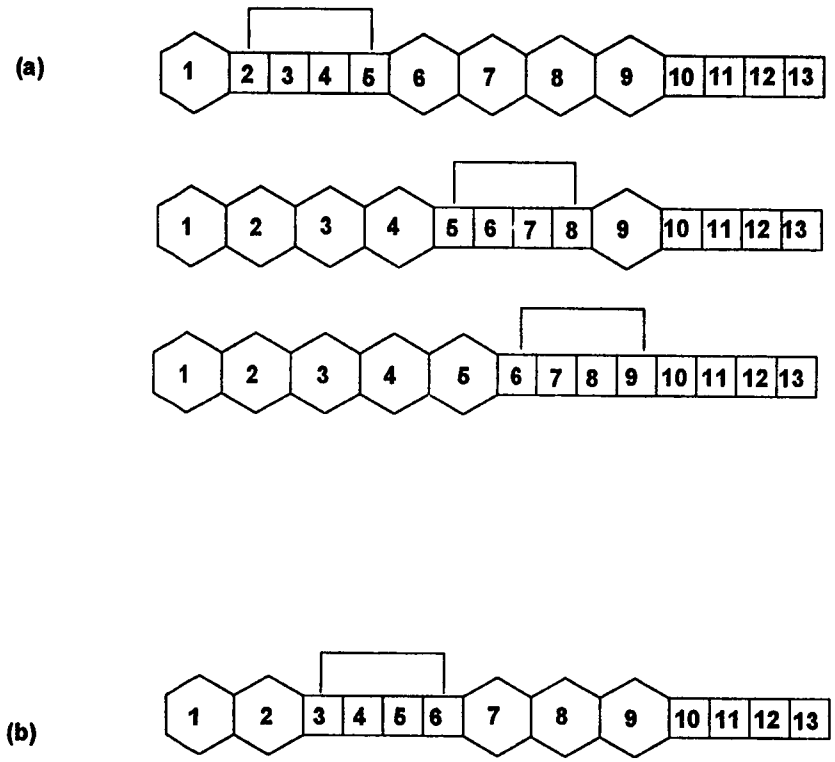


Figure 8.1 Proposal for helix propagation in the cyclic Dyn A-(1-13)NH_2 analogues
(a) a helix may propagate from residues 1 to 9
(b) a helix may not be able to propagate because the position of the constraint prevents helix propagation.

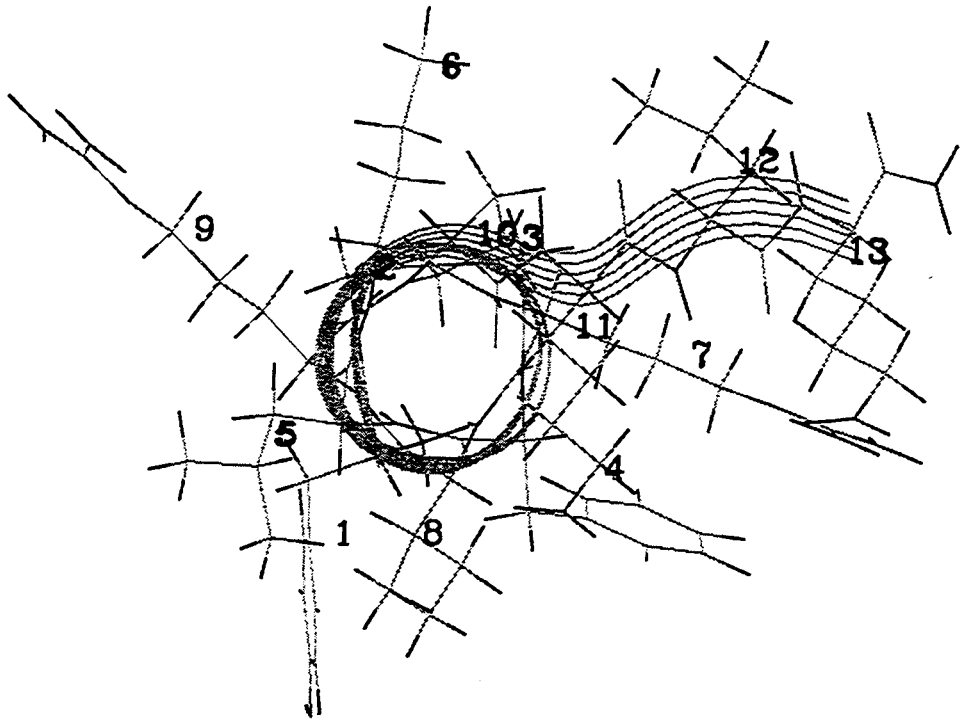


Figure 8.2 Helical wheel of Dynorphin A-(1-13)NH_2

According to these speculations, possible future work in this area is as follows:

1. Examination of possible conformations of all of the cyclic [D-Asp^{i},Dap^{i+3}] Dyn A-(1-13)NH_2 analogues using NMR, since NMR can provide information concerning whether helical structure is present and if so where.

2. Design a more rigid scaffold that can initiate or stabilize a helical structure in Dyn A molecule. Bicyclic Dyn A analogues containing constraints at both positions [2,5] and [6,9] may be suitable to form this more rigid molecule.

3. Investigate the effects of stereochemistry and/or other side chain functionality at residue 6 of Dyn A.

The second objective in this study involved the synthesis of a lactam. Since the cyclization reaction is generally the yield-limiting step, evaluation of activating reagents used for the formation of a lactam was therefore important. In this study, it was found that yield and purity of the desired cyclic peptide was dependent upon the activating reagent used. The uronium reagents, HBTU, HATU and HAPyU, all consistently gave alkylguanidinium byproducts which resulted in low yields of the desired cyclic peptides. Although the phosphonium reagents BOP, PyBOP and PyAOP gave slow cyclization reactions, these reagents proved to be useful in the production of a lactam, particularly when the cyclization was difficult. Since there are other factors which may affect the yield and purity of the desired cyclic peptide obtained from the cyclization reaction, further study could provide additional information on this key reaction. These include:

1. A study of solvents and auxilliary solvents which may affect the secondary structure of the peptide.

2. Different chemistries for the solid support, particularly the new polyethylene glycol-polystyrene resin, which can be used in a wide range of solvents, could be examined.

The last project involved analytical methods which are useful for monitoring peptide synthesis. In this study, HPLC with on-line mass spectrometry and tandem mass spectrometry proved to be very powerful and convenient methods for evaluating the variety of peptide products obtained from various cyclization reactions. These methods provided sufficient information to identify peptides in either pure or crude forms. In terms of the information obtained and savings in time and labor, the advantages of these two mass spectrometric methods can more than compensate for the high cost of these analyses.

BIBLIOGRAPHY

Al-Obeidi, F.; Castrucci, A.M.L.; Hadley, M.E.; Hruby, V.J. Potent and Prolonged Acting Cyclic Lactam Analogues of α-Melanotropin: Design Based on Molecular Dynamics. *J. Med. Chem.* **1989**, *32*, 2555-2561.

Albericio, F,; Kneib-Cordonier, N.; Biancalana, S.; Gera, L.; Masada, R.I.; Hudson, D.; Barany, G. Preparation and Aplication of the 5-(4-(9-fluorenylmethoxy carbonyl)aminomethyl-3,5-dimethoxyphenoxy)valeric Acid (PAL) Handle for the Solid-Phase Synthesis of C-terminal Peptide Amides under Mild Conditions *J. Org. Chem.* **1990**, *55*, 3730-3743.

Anwer, M.K.; Spatola, A.F. An Advantageous Method for the Rapid Removal of Hydrogenolysable Protecting Groups under Ambient Conditions; Synthesis of Leucine-enkephalin. *Synthesis* **1980**, 929-932.

Arttamangkul, S.; Murray, T.F.; DeLander, G.E.; Aldrich, .J.V. Synthesis and Opioid Activity of Conformationally Contrained Dyn A Analogues. Part I. The Constrained in the "Message" Sequence. *J. Med. Chem.* (submitted).

Arunlakshana, O.; Schild, H.O. Some Quantitative Uses of Drug Antagonists. *Br. J. Pharmacol. Chemother.* **1959**, *14*, 48-58.

Atherton, E.; Sheppard, R.C. The Fluorenylmethoxycarbonyl Amino Protecting Group. In *The Peptides. Vol.9* Udenfriend, S,; Meienhofer, J., Eds.; Academic Press: New York, 1987, pp 1-38.

Atherton, E.; Sheppard, R.C. In *Solid Phase Peptide Synthesis, A Practical Approach.* Atherton, E.; Sheppard, R.C., Eds.; IRL Press: Oxford, 1989a, pp 13-23.

Atherton, E.; Sheppard, R.C., 1989b, ibid pp 25-37.

Atherton, E.; Sheppard, R.C., 1989c, ibid pp 39-45.

Attali, B.; Guarderes, C.; Mazurguil, H.; Audigier, Y.; Cros, J. Evidence for Multiple Kappa Binding-Sites by Use of Opioid Peptides in the Guinea-Pig Lumbo-Sarcral Spinal-Cord. *Neuropeptid* **1982**, *3*, 53-64.

Auzanneau, F.-I.; Meldal, M.; Bock, K. Synthesis, Characterization and Biocompatibility of PEGA Resins. *J. Peptide Sci.* **1995**, *1*, 31-44.

Barany, G.; Albericio, F.; Solé, N.A.; Griffin, G.W.; Kates, S.A.; Hudson, D. Novel Polyethylene Glycol-polystyrene (PEG-PS) Graft Supports for Solid-Phase Peptide Synthesis. *Peptides*. **1992**, 267-268.

Barany,G.; Jneib-Cordonier, N.; Mullen, D.G. Solid-Phase Peptide Synthesis: A Silver Anniversary Report. *Int. J. Peptide Protein Res.* **1987**, *30*, 705-739.

Barany, G.; Merrifield, R.B. Solid-Phase Peptide Synthesis. In *The Peptides, Vol.2*, Gross, E.; Meienhofer, J., Eds.; Academic Press: New York, 1979, pp 1-284.

Barnard, E.A.; Simon, J. Opioid Receptors. In *The New Comprehensive Biochemistry Vol. 24: Neurotransmitter Receptors*, Hucho, F. Ed.; Elsevier: New York, 1993, pp 297-323.

Beck-Sickinger, A.G.; Schnorrenberg, G.; Metzger, J.; Jung, G. Sulfonation of Arginine Residues as Side Reaction in Fmoc-Peptide Synthesis. *Int. J. Peptide Protein Res.* **1991**, *38*, 25-31.

Benoiton, N.L. Quantitation and Sequence Dependence of Racemization in Peptide Synthesis. In *The Peptides, Vol. 5,* Gross, E.; Meienhoffer, J., Eds; Academic Press: New York, 1983, pp 217-284.

Biemann, K. Mass Spectrometric Methods for Protein Sequencing. *Anal. Chem.* **1986**, *58*, 1288A-1300A.

Biemann, K. Contributions of Mass Spectrometry to Peptide and Protein Structure. *Biomed. Environ. Mass Spectrom.* **1988**, *16*, 99-111.

Biemann, K.; Martin, S.A. Mass Spectrometric Determination of the Amino Acid Sequence of Peptides and Proteins. *Mass Spectrom. Rev.* **1987**, *6*, 1-76.

Biemann, K.; Scoble, H. Characterization by Tandem Mass Spectrometry of Structural Modifications in Proteins. *Science* **1987**, *237*, 992-998.

Bodanszky, M. Tactics and Strategy in Peptide Synthesis. In *Principles of Peptide Synthesis*, Bodanszky, M. Ed.; Spriner-Verlag: New York, 1993, pp 215-247.

Bodanszky, M.; Bodanszky, A. *The Practice of Peptide Synthesis*; Springer-Verlag: New York, **1984**; p 20.

Bodanszky, M.; Martinez, J. Side Reactions in Peptide Synthesis. In *The Peptides, Vol. 5,* Gross, E.; Meienhoffer, J., Eds; Academic Press: New York, 1983, pp 111-216.

Bonnett, K.A.; Groth, J.; Gioannini, T.; Cortes, M.; Simon, E.J. Opiate Receptor Heterogeneity in Human Brain Regions. *Brain Res.* **1981**, *221*, 431-440.

Bracken, C.; Gulyás, J.; Taylor, J.W.; Baum, J. Synthesis and Nuclear Magnetic Resonance Structure Determination of an α-Helical, Bicyclic, Lactam-Bridged Hexapeptide. *J. Am. Chem. Soc.* **1994**, *116*, 6431-6432.

Bradley, E.K.; Thomason, J.F.; Cohen, F.E.; Kosen, P.A.; Kuntz, I.D. Studies of Synthetic Helical Peptides Using Circular Dichroism and Nuclear Magnetic Resonance. *J. Mol. Biol.* **1990**, *215*, 607-622.

Brantl, V.; Teschemacher, H.; Bläsig, J.; Henschen, A.; Lottspeich, F. Opioid Activities of β-Casomorphins. *Life Sci.* **1981**, *28*, 1903-1909.

Brownstein, M.J. A Brief History of Opiates, Opioid Peptides, and Opioid Receptors. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5391-5393.

Bunzow, J.R.; Zhang, G.; Bouvier, C.; Saez, C.; Ronnekleiv, O.K.; Kelly, M.J.; Grandy, D.K. Characterization and Distribution of a Cloned Rat μ-Opioid Receptor. *J. Neurochem.* **1995**, *64*, 14-24.

Carpino, L.A. 1-Hydroxy-7-Azabenzotriazole. An Efficient Peptide Coupling Additive. *J. Am. Chem. Soc.* **1993**, *115*, 4397-4398.

Carpino, L.A.; Chao, H.G. (9-Fluorenylmethyl)oxy)carbonyl Amino Acid Chlorides in Solid-Phase Peptide Synthesis. *J. Org. Chem.* **1991**, *56*, 2635-2642.

Carpino, L.A.; El-Faham, A.; Albericio, F. Racemization Studies During Solid-Phase Peptide Synthesis Using Azabenzotriazole-based Coupling Reagents. *Tetrahedron Lett.* **1994a**, *35*, 2279-2282.

Carpino, L.A.; El-Faham, A.; Minor, C.A.; Albericio, F. Advantages Applications of Azabenzotriazole(Triazolopyridine)-based Coupling Reagents to Solid-Phase Peptide Synthesis. *J. Chem. Soc., Chem. Commun.* **1994b**, 201-203.

Carpino, L.A.; Han, G.Y. The 9-Fluorenylmethoxycarbonyl Amino-Protecting Group. *J. Med. Chem.* **1972**, *37*, 3404-3409.

Carpino, L.A.; Mansour, E.M.E.; Sadat-Aalaee, D. *tert*-Butyloxycarbonyl and Benzyloxycarbonyl Amino Acid Fluorides. New, Stable, Rapid-Acting Acylating Agents for Peptide Synthesis. *J. Org. Chem.* **1991**, *56*, 2611-2614.

Carpino, L.A.; Sadat-Aalaee, D.; Chao, H.G.; DeDelms, R.H. ((9-Fluorenylmethyl)-oxy)carbonyl (FMOC) Amino Acid Chlorides. Convenient New Peptide

Coupling Reagents Applicable to the FMOC/*tert*-Butyl Strategy for Solution and Solid-Phase Syntheses. *J. Am. Chem. Soc.* **1990**, *112*, 9651-9652.

Castro B.; Dormoy, J.-R.; Evin, G.; Selve, C. Reactifs De Couplage Peptidique IV (1)-L'Hexafluorophosphate De Benzotriazolyl N-Oxytrisdimethylamino Phosphonium (B.O.P.). *Tetrahedron Lett.* **1975**, *14*, 1219-1222.

Chakrabartty, A.; Kortemme, T.; Padmanabhan, S.; Baldwin, R.L. Aromatic side-Chain Contribution to Far-Ultraviolet Circular Dichroism of Helical Peptides and Its Effect on Measurement of Helix Propensities. *Biochemistry* **1993**, *32*, 5560-5565.

Chang, K.-J. Opioid Receptors: Multiplicity and Sequelae of Ligand-Receptor Interactions. In *The Receptors Vol. I*, Conn, P.M., Ed.; Academic Press, Inc.: New York, 1984, pp 2-81.

Chang, K.-J.; Cooper, B.R.; Hazum, E.; Cuatrecasas, P. Multiple Opiate Receptors: Different Regional Distribution in the Brain and Differential Binding of Opiates and Opioid Peptides. *Mol. Pharmacol.* **1979**, *16*, 91-104.

Chang, K.-J.; Cuatrecasas, P. Multiple Opiate Receptors: Enkephalins and Morphine Bind to Receptors of Different Specificity. *J. Biol. Chem.* **1979**, *254*, 2610-2618.

Chang, K.-J.; Killian, A.; Hazum, E.; Cuatrecasas, P.; Chang, J.-K. Morphiceptin: A Potent and Specific agonist for Morphine (μ) Receptors. *Science* **1981**, *212*, 75-77.

Chavkin, C.; Goldstein, A. Specific Receptor for the Opioid Peptide Dynorphin: Structure-Activity Relationships. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 6543-6547.

Chavkin, C.; James, I.F.; Goldstein, A. Dynorphin Is a Specific Endogenous Ligand of the κ Opioid Receptor. *Science* **1982**, *215*, 413-415.

Chen, Y.; Mestek, A.; Liu, J.; Hurley, J.A.; Yu, L. Molecular Cloning and Functional Expression of a μ-Opioid Receptor form Rat Brain. *Mol. Pharmacol.* **1993a**, *44*, 8-12.

Chen, Y.; Mestek, A.; Liu, J.; Yu, L. Molecular Cloning of a Rat κ Opioid Receptor Reveals sequence similarities to the μ and δ Opioid Receptors. *Biochem. J.* **1993b**, *295*, 625-628.

Chen, G.C., Yang, J.T. Two-Point Calibration of Circular Dichrometer with d-10-Camphorsulfonic Acid *Anal. Lett.* **1977**, *10*, 1195-1207.

Cheng, Y.-C.; Prusoff, W.H. Relationship Between the Inhibition Constant (K_I) and the Concentration of Inhibitor Which Causes 50 Per Cent Inhibition (I_{50}) of an Enzymatic Reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099-3108.

Choi, H.; Aldrich, J.V. Comparison of Methods for the Fmoc Solid-Phase Synthesis and Cleavage of a Peptide Containing Both Tryptophan and Arginine. *Int. J. Peptide Protein Res.* **1993**, *42*, 58-63.

Choi, H.; Murray, T.F.; DeLander, G.E.; Caldwell, V.; Aldrich, J.V. N-Terminal Alkylated Derivatives of [D-Pro10]Dynorphin A-(1-11) Are Highly Selective for κ-Opioid Receptor. *J. Med. Chem.*, **1992**, *35*, 4638-4639.

Clark, J.A.; Liu, L.; Price, M.; Hersh, B.; Edelson, M.; Pasternak, G.W. Kappa Opiate Receptor Multiplicity: Evidence for Two U50-488-sensitive κ_1 Subtypes and a Novel κ_3 Subtypes. *J. Pharmacol. Exp. Ther.* **1989**, *251*, 461-468.

Cohen, M.L.; Mendelsohn, L.G.; Mitch, C.H.; Zimmerman, D.M. Use of the Mouse Vas Deferens to Determine μ, δ and κ Receptor Affinities of Opioid Antagonists. *Receptor* **1994**, *4*, 43-53.

Colburn, J.C.; Grossman, P.D.; Moring, S.E.; Lauer, H.H. Analysis of Proteins and Peptides by Free Solution Capillary Electrophoresis (CE). In *HPLC of Peptides and Proteins: Separation, Analysis, and Conformation*, Mant, C.T.; Hodges, R.S., Eds.; CRC Press: Boston, 1991; pp 895-901.

Corbett, A.D.; Paterson, S.J.; McKnight, A.T.; Magnan, J.; Kosterlitz, H.W. $Dynorphin_{1-8}$ and $Dynorphin_{1-9}$ are Ligands for the κ-Subtypes of Opiate Receptor. *Nature (London)* **1982**, *299*, 79-81.

Coste, J.; Dufour, M.-N.; Le-Nguyen, D.; Castro, B. BOP and Congeners: Present Status and New Developments. In *Peptides: Chemistry and Biology*, Smith, J.A.; Rivier, J.E., Eds.; Escom: Leiden, The Netherlands, 1992, pp 885-888.

Coste, J.; Frérot, E.; Jouin, P. Coupling *N*-methylated Amino Acids Using PyBroP and PyCloP Halogenophosphonium Salts: Mechanism and Fields of Application. *J. Org. Chem.* **1994**, *59*, 2437-2446.

Coste, J.; Le-Nguyen, D.; Castro, B. PyBOP®: A New Peptide Coupling Reagent Devoid of Toxic By-Product. *Tetrahedron Lett.* **1990**, *31*, 205-208.

Cox, B.M.; Ophelm, K.E.; Teschemacher, H.; Goldstein, A. A Peptide-like Substance from Pituitary that Acts Like Morphine. 2. Purification and Properties. *Life Sci.* **1975**, *16*, 1777-1782.

Dale, J. Conformational Aspects of Many-Membered Rings. *Angew. Chem Int. Ed. Engl.* **1966**, *24*, 1070-1093.

Donnerer, J.; Oka, K.; Brossi, A.; Rice, K.C.; Spector, S. Presence and Formation of Codeine and Morphine in the Rat. *Proc. Natl. Acad. Sci.* **1986**, *83*, 4566-4567.

Dourtoglou, V.; Gross, B. *O*-(benzotriazol-1-yl)-*N,N,N",N"*-tetramethyluronium hexafluorophosphate as Coupling Reagent for the Synthesis of Peptides of Biological Interest. *Synthesis* **1984**, 572-574.

Dourtoglou, V.; Ziegler, J.C.; Gross, B. L´ Hexafluorophosphate De *O*-Benzotriazolyl-*N,N*-tetramethyluronium: Un Reactif De Couplage Peptidique Nouveau Et Efficace. *Tetrahedron Lett.* **1978**, 1269.

Ehrlich, A.; Rothemund, S.; Brudel, M.; Beyermann, M.; Carpino, L.A.; Bienert, M. Synthesis of Cyclic Peptides via Efficient New Coupling Reagents. *Tetrahedron Lett.* **1993**, *34*, 4781-4784.

Elwell, M.L.; Schellmen, J.A. Stability of Phage T4 Lysosymes 1. Native Properties and Thermal Stability of Wild Type and Two Mutant Lysosymes. *Biochim. Biophys. Acta* **1977**, *494*, 367-383.

Erspamer, V. The Opioid Peptides of the Amphibian Skin. *Int. J. Devl. Neuroscience* **1992**, *10*, 3-30.

Erspamer, V.; Melchiorri, P.; Falconieri Erspamer, G.; Negri, L.; Corsi, R.; Severini, C.; Barra, D.; Simmaco, M.; Kreil, G. Deltorphins: a Family of Naturally Occurring Peptides with High Affinity and Selectivity for δ-Opioid Binding Sites. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5188-5192.

Evans, C.J.; Keith, D.E. Jr.; Morrison, H.; Magendzo, K.; Edwards, R.H. Cloning of a Delta Opioid Receptor by Functional Expression. *Science* **1992**, *258*, 1952-1955.

Felix, A.M.; Heimer, E.P.; Wang, C.-T.; Lambros, T.J.; Fournier, A.; Mowles, T.F.; Maines, S.; Campbell, R.M.; Wegrzynski, B.B.; Toome, V.; Fry, D.; Madison, V.S. Synthesis, Biological Activity and Conformational Analysis of Cyclic GRF Analogs. *Int. Peptide Protein Res.* **1988a**, *32*, 441-454.

Felix, A.M.; Wang, C.-T.; Heimer, E.P.; Fournier, A. Applications of BOP Reagent in Solid Phase Synthesis. II. Solid Phase Side-chain to Side-chain cyclizations Using BOP Reagent. *Int. J. Peptide Protein Res.* **1988b**, *31*, 231-238.

Fields, C.G.; Lloyd, D.H.; Macdonald, R.L.; Otteson, K.M.; Noble, R.L. HBTU Activation for Automated Fmoc Solid-Phase Peptide Synthesis. *Peptide Res.* **1991**, *4*, 95-101.

Fields, G.B.; Tian, Z.; Barany, G. Principles and Solid-Phase Peptide Synthesis. In *Synthetic Peptides, A User's Guide.* Grant, G.A., Ed.; W.H. Freeman and Company: New York, 1992, pp 77-183.

Fischli, W.; Goldstein, A.; Hunkapiller, M.; Hood, L. Isolation and Amino Acid Sequence Analysis of a 4,000-dalton Dynorphin from Porcine Pituitary. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 5435-5437.

Fournier, A.; Danho, W.; Felix, A.M. Applications of BOP Reagent in Solid Phase Synthesis. III. Solid Phase Peptide Synthesis with Unprotected Aliphatic and Aromatic Hydroxyamino Acids Using BOP Reagent. *Int. J. Peptide Protein Res.* **1989**, *33*, 133-139.

Fournier, A.; Wang, C.-T.; Felix, A.M. Applications of BOP Reagent in Solid Phase Synthesis. Advantages of BOP Reagent for Difficult Couplings Exemplified by a Synthesis of [Ala^{15}]-GRF(1-29)-NH_2. *Int. J. Peptide Protein Res.* **1988**, *31*, 86-97.

Fries, D.S. Analgesic Agonists and CNS Receptors. *Advances in CNS Drug-Receptor Interactions* **1991**, *1*, 1-21.

Fuji, N.; Otaka, A.; Ikemura, O.; Akaji, K.; Funakoshi, S.; Hayashi, Y.; Kuroda, Y.; Yajima, H. Trimethylsilyl Trifluoromethanesulfonate as a Useful Deprotecting Reagent in Both Solution and Solid Phase Peptide Synthesis. *J. Chem. Soc. Chem. Commun.* **1987**, 274-275.

Fukudome, S.-I.; Yoshikawa, M. Gluten Exorphin C. A Novel Opioid Peptide Derived from Wheat Gluten. *FEBS Lett.* **1993**, *316*, 17-19.

Fuller, W.D.; Cohen, M.P.; Shabankareh, M.; Blair, R.K.; Goodman, M.; Naider, F.R. Urethane-Protected Amino Acid N-Carboxy Anhydrides and Their Use in Peptide Synthesis. *J. Am. Chem. Soc.* **1990**, *112*, 7414-7416.

Gairin, J.E.; Gouarderes, C.; Mazarguil, H.; Alvinerie, P.; Cros, J. [D-Pro^{10}]Dynorphin-(1-11) Is a Highly Potent and Selective Ligand of κ Opioid Receptors. *Eur. J. Pharmacol.* **1984**, *106*, 457-458.

Gausepohl, H.; Kraft, M.; Frank, R. *In Situ* Activation of Fmoc-Amino Acids by BOP in Solid Phase Peptide Synthesis. In *Peptides: Chemistry and Biology* Jung, G.; Bayer, E., Eds.; Water de Gruyter and Co.: Berlin, Germany, 1989, pp 523-524.

Gausepohl, H,; Pieles, U.; Frank, R.W. Schiff's Base Analog Formation During *In Situ* Actvation by HBTU and TBTU. In *Peptides: Chemistry and Biology* Smith, J.A.; Rivier, J.E., Eds.; ESCOM: Leiden, The Netherlands, 1992, pp 523-524.

Geiger, T.; Clarke, S. Deamination, Isomerization, and Racemization at Asparaginyl and Aspartyl Residues in Peptides. *J. Biol. Chem.* **1987**, *262*, 785-794.

Ghadiri, M.R.; Choi, C. Secondary Structure Nucleation in Peptides. Transition Metal Ion Stabilized α-helices. *J. Am. Chem.* **1992**, *112*, 1630-1632.

Gilon, C.; Halle, D.; Chorev, M.; Selinger, Z.; Byk, G. Backbone Cyclization: A New Method for Conferring Conformation Constraint on Peptides. *Biopolymer* **1991**, *31*, 745-750.

Gioannini, T.L.; Howard, A.D.; Hiller, J.M.; Simon. E.J. Purification of an Active Opioid-binding Protein from Bovine Striatum. *J. Biol. Chem.* **1985**, *260*, 15117-15121.

Goldstein, A.; Fischli, W.; Lowney, L.I.; Hunkapiller, M.; Hood, L. Porcine Pituitary Dynorphin: Complete Amino Acid Sequence of the Biologically Active Heptadecapeptide. *Proc. Natl. Acad.Sci. U.S.A.* **1981**, *78*, 7219-7223.

Goldstein, A.; James, I.F. Site-Directed Alkylation of Multiple Opioid Receptors. II. Pharmacological Selectivity. *Mol. Pharmacol.* **1984**, *25*, 343-348.

Goldstein, A.; Naidu, A. Multiple Opioid Receptors: Ligand Selectivity Profiles and Binding Site Signatures. *Mol. Pharmacol.* **1989**, *36*, 265-272.

Goldstein, A.; Tachibana, J.S.; Lowney, L.I.; Hunkapiller, M.; Hood, L. Dynorphin-(1-13), an Extraordinarily Potent Opioid Peptide. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 6666-6670.

Grant, G.A. Synthetic Peptides: Spanning the Twentieth Century. In *Synthetic Peptides, A User's Guide*. Grant, G.A., Ed.; W.H. Freeman and Company: New York, 1992, pp 1-7.

Gross, E.; Meienhoffer, J. The Peptide Bond. In *The Peptides, Vol.1*, Gross, E.; Meienhofer, J., Eds.; Academic Press: New York, 1979, pp 1-64.

Handa. B.K.; Lane, A.C.; Lord, J.A.H.; Morgan, B.A.; Rance, M.J.; Smith, C.F.C. Analogues of β-LPH$^{61-64}$ Possessing Selective Agonist Activity at μ-Opiate Receptors. *Eur. J. Pharmacol.* **1981**, *70*, 531-540.

Hansen, P.E.; Morgan, B.A. Structure-Activity Relationships in Enkephalin Peptides. In *The Peptides, Vol 6.* Udenfriend, S.; Meienhofer, J., Eds,; Academic Press: New York, 1984, pp 269-321.

Hay, R.W.; Morris, P.J. Proton Ionisation Constants and Kinetics of Base Hydrolysis of Some α-Amino-acid Esters in Aqueous Solution. Part III. Hydrolysis and Intramolecular Aminolysis of $\alpha\omega$-Diamino-acid Methyl Esters. *J. Chem. Soc. Perkin II* **1972**, 1021-1029.

Hernandez, J.-F; Kornreich, W.; Rivier, C.; Miranda, A.; Yamamoto, G.; Andrews, J.; Taché, Y.; Vale, W.; Rivier, J. Synthesis and Relative Potencies of New Constrained CRF Antagonists. *J. Med. Chem.* **1993**, *36*, 2860-2867.

Hoffmann, E.; Beck-Sickinger, A.G.; Jung, G. Investigations on Different Coupling Reagents for the Synthesis of a Cyclopeptide Analog of Neuropeptide Y. *Liebigs Ann. Chem.* **1991**, 585-590.

Hruby, V.J. Conformational Restrictions of Biologically Active Peptides via Amino Acid Side Chain Groups. *Life Sci.* **1982**, *31*, 189-199.

Hruby, V.J.; Al-Obeidi,F.; Kazmierski, W. Emerging Approaches in the Molecular Design of Receptor-Selective Peptide Ligands: Conformational, Topographical and Dynamic Considerations. *Biochem. J.* **1990**, *268*, 249-262.

Hudson, D. Methodological Implications of Simultaneous Solid-Phase Peptide Synthesis 1: Comparison of Different Coupling Procedures. *J. Org. Chem.* **1988**, *53*, 617-624.

Hughes, J. Redlections on Opioid Peptides. In *Opioids: Past, Present and Future.* Hughes, J.; Collier, H.O.J.; Rance, M.J.; Tyers, M.B., Eds.; Taylor & Francis: London, 1984, pp 9-19.

Hughes, J.; Smith, T.W.; Kosterlitz, H.W.; Fothergill, L.A.; Morgan, B.A.; Morris, H.R. Identification of Two Related Pentapeptides from the Brain with Potent Opiate Agonist Activity. *Nature (London)*, **1975**, *258*, 577-579.

Itzhak, Y.; Hiller, J.M.; Simon, E.J. Solubilization and Characterization of μ, δ and κ Opioid Binding Sites from Guinea Pig Brain: Physical Separation of κ Receptors. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 4217-4221.

Jaffe, J.H.; Martin, W.R. Opioid Analgesics and Antagonist. In *The Pharmacological Basis of Therapeutics*, 8th ed.; Gilman, A.G., Goodman, L.S., Rall, T.W., Niles, A.s., Taylor, P., Eds.; Pergamon Press: New York, 1990; pp 485-521.

James, I.F. Opioid Receptors for the Dynorphin Peptides. *Natl. Inst. Drug Abuse Res. Monogr.* **1986**, *70*, 192-208.

James, I.F., Fischli, W.; Goldstein, A. Opioid Receptor Selectivity of Dynorphin Gene Products. *J. Pharmacol. Exp. Ther.* **1984**, *228*, 88-93.

James, I.F.; Goldstein, A. Site-Directed Alkylation of Multiple Opioid Receptors. I. Binding Selectivity. *Mol. Pharmacol.* **1984**, *25*, 337-342.

Johnson, R.S.; Martin, S.A.; Biemann, K.; Stults, J.T.; Watson, J.T. Novel Fragmentation Process of Peptides by Collision-Induced Decomposition in a Tandem Mass Spectrometer: Differentiate of Leucine and Isoleucine. *Anal. Chem.*, **1987**, *59*, 2621-2625.

Kallick, D. Conformation of Dynorphin A(1-17) Bound to Dodecylphosphocholine Micelles. *J. Am. Chem. Soc.* **1993**, *115*, 9317-9318.

Kates, S.A.; Daniels, S.B.; Albericio, F. Automated Allyl Cleavage for Continuous-Flow Synthesis of Cyclic and Branched Peptides. *Anal. Biochem.* **1993**, *212*, 303-310.

Kates, S.A.; Solé, N.A.; Albericio, F.; Barany, G. Solid-Phase Synthesis of Cyclic Peptides. In *Peptides: Design, Synthesis and Biological Activity*, Basava, C.; Anantharamaiah, G.M., Eds.; Birkhäuser: Boston, 1994, pp 39-58.

Kawasaki, A.M.; Knapp, R.J.; Kramer, T.H.; Wire, W.S.; Vasquez, O.S.; Yamamura, H.I.; Burks, T.F.; Hruby, V.J. Design and Synthesis of Highly Potent and Selective Cyclic Dynorphin A Analogs. *J. Med. Chem.* **1990**, *33*, 1874-1879.

Kawasaki, A.M.; Knapp, R.J.; Kramer, T.H.; Walton, A.; Wire, W.S.; Hashimoto, S.; Yamamura, H.I.; Porreca, F.; Burks, T.F.; Hruby, V.J. Design and Synthesis of Highly Potent and Selective Cyclic Dynorphin A Analogs. 2. New Analogs. *J. Med. Chem.* **1993a**, *36*, 750-757.

Kawasaki, A.M.; Knapp, R.J.; Walton, W.S.; Zalewska, T.; Yamamura, H.I.; Porreca, F.; Burks, T.M.; Hruby, V.J. Syntheses, Opioid Binding Affinities, and Potencies of Dynorphin A Analogues Substituted in Position 1, 6, 7, 8 and 10. *Int. J. Pept. Protein Res.* **1993b**, *42*, 411-419.

Kemp, D.S.; Curran, T.P. (2S,5S,8S,11S)-1-Acetyl-1,4-diaza-3-keto-5-carboxy-10-thia-tricyclo-[2.8.0]-tridecane, **1**, Synthesis of Prolyl-proline-derived, Peptide-functionalized Templates for α-Helix Formation. *Tetrahedron Lett.* **1988**, *29*, 4931-4934.

Kessler, H. Conformation and Biological Activity of Cyclic Peptides. *Angew. Chem. Int. Ed. Engl.* **1982**, *21*, 512-523.

Kessler, H.; Haase, B. Cyclic Hexapeptides Derived from the Human Thymopoietin III. *Int. J. Peptide Protein Res.* **1992**, *39*, 36-40.

Kieffer, B.L.; Befort, K.; Gaveriaux-Ruff, C.; Hirth, C.G. The δ-Opioid Receptor: Isolation of a cDNA by Expression Cloning and Pharmacological Characterization. *Proc. Natl. Acad. Sci. USA.* **1992**, *89*, 12048-12052.

Kim, H.Y.; Pilosof, D.; Dyckes, D.F; Vestal, M.L. On-Line Peptide Sequencing by Enzymatic Hydrolysis, High Performance Liquid Chromatography, and Thermospray Mass Spectrometry. *J. Am. Chem. Soc.* **1984**, *106*, 7304-7309.

King, D.S.; Fields,C.G.; Fields, G.B. A Clevage Method for Minimizing Side Reactions Following Fmoc Solid Phase Peptide Synthesis. *Int. J. Peptide Protein Res.* **1990**, *36*, 255-266.

Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillessen, D. New Coupling Reagents in Peptide Chemistry. *Tetrahedron Lett.* **1989**, *30*, 1927-1930.

König, W. Opioid Peptides. In *Peptide and Protein Hormones: Structure, Regulation, Activity. A Reference Manual.* VCH: New York, 1993, pp 69-88.

König, W.; Geiger, R. Eine neue Methode zur Synthese von Peptiden: Aktivierung der Carboxylgruppe mit Dicyclohexylcarbodiimid unter Zusatz von 1-hydroxybensotriazolen. *Chem. Ber.* **1970**, *103*, 788-798.

Kosterlitz, H.W.; Waterfield, A.A. *In Vitro* Models in the Study of Structure-Activity Relationships of Narcotic Analysis. *Ann. Rev. Pharmacol. Toxicol.* **1975**, *15*, 29-47.

Kramer, T.H.; Shook, J.E.; Kazmierski, W.; Ayres, E.A.; Wire, W.S.; Hruby, V.J.; Burks, T.F. Novel Peptidic *Mu* Opioid Antagonists: Pharmacological Characterization *in Vitro* and *in Vivo*. *J. Pharmacol. Exp. Ther.* **1989**, *249*, 544-551.

Krishnamurthy, T.; Szafraniec, L.; Hunt, D.F.; Shabanowitz, J.; Yates III, J.R.; Hauer, C.R.; Carmichael, W.W.; Skulberg, O.; Codd, G.A.; Missler, S.

Structure Characterization of Toxic Cyclic Peptides from Blue-Green Algae by Tandem Mass Spectrometry. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 770-774.

Le-Nguyen, D.; Heitz, A.; Castro, B. Renin Substrates. Part 2. Rapid Solid Phase Synthesis of the Ratine Sequence Tetradecapeptide Using BOP Reagent. *J. Chem. Soc. Perkin Trans.* **1987**, 1915-1919.

Le-Nguyen, D.; Seyer, R.; Heitz, A.; Castro, B. Renin Subtrates. Part 1. Liquid-phase Synthesis of the Equine Sequence with Benzotriazolyloxytris-(dimethylamino)phosphonium Hexafluorophosphate (BOP). *J. Chem. Soc. Perkin Trans.* **1985**, 1025-1031.

Lemaire, S.; Lafrance, L.; Dumont, M. Synthesis and Biological Activity of Dynorphin-(1-13) and Analogs Substituted in Positions 8 and 10. *Int. Peptide Protein Res.* **1986**, *27*, 300-305.

Leslie, F.M. Methods Used for the Study of Opioid Receptors. *Pharmacol. Rev.* **1987**, *39*, 197-249.

Lloyd, J.R.; Cotter, M.L.; Ohori, D.; Doyle, D.L. Distinction of α- and β- Aspartyl and α- and γ-Glutamyl Peptides by Fast Atom Bombardment/Tandem Mass Spectrometry. *Biomed. Environ. Mass Spectrom.* **1988**, *15*, 399-402.

Lloyd-Williams, P.; Albericio, F.; Giralt, E. Convergent Solid-Phase peptide Synthesis. *Tetrahedron* **1993**, *49*, 11065-11133.

Loffet, A.; Zhang, H.X. Allyl-based Groups for Side-chain Protection of Amino Acids. *Int. J. Peptide Protein Res.* **1993**, *42*, 346-351.

Lord, J.A.H.; Waterfield, A.A.; Hughes, J.; Kosterlitz, H.W. Endogenous Opioid Peptides: Multiple Agonists and Receptors. *Nature (London)* **1977**, *267*, 495-499.

Lung, F.-D.; Collins, N.; Lou, B.-S.; Meyer, J.-P.; Li, G.; Stropova, D.; Porecca, F.; Yamamura, H.I.; Hruby, V.J. Poster P296: Synthesis, Opioid Activities, and Binding Affinities of Dynorphin A Analogues with Position-3 Conformation Constraints: New Insights into Requirements for κ Receptors. 23rd European Peptide Symposium, September 4 -10, 1994, Braga, Portugal.

Mann, M.; Meng, C.K.; Fenn, J.B. Interpreting Mass Spectrometry to Peptide and Protein Structure. *Biomed. Environ. Mass Spectrom.* **1989**, *61*, 1702 -1708.

Maroun, R.; Mattice, W.L. Solution Conformations of the Pituitary Opioid Peptide Dynorphin A(1-13). *Biochem. Biophys. Res. Commun.* **1981**, *103*, 442-446.

Marqusee, S.; Baldwin, R.L. Helix Stabilization by Glu^----Lys^+ Salt Bridges in Short Peptides of *De Novo* Design. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 8898-8902.

Martin, W.R.; Eades, C.G.; Thompson, J.A.; Huppler, R.E.; Gilbert, P.E. The Effects of Morphine and Nalorphine Like Drugs in the Nondependent and Morphine-Dependent Chronic Spinal Dog. *J. Pharmacol. Exp. Ther.* **1976**, *197*, 517-532.

McKnight, A.T.; Corbett, A.D.; Marcoli, M.; Kosterlitz, H.W. Hamster Vas Deferens Contains δ-Opioid Receptors. *Neuropeptides* **1984**, *5*, 97-100.

McKnight, A.T.; Rees, D.C. Opioid Receptors and Their Ligands. *Neurotransmissions* **1991**, *7*, 1-6.

McMurray, J.S.; Lewis, C.A.; Obeyesekere, N.U. Influence of Solid Support, Solvent and Coupling Reagent on the Head-to-Tail Cyclization of Resin-Bound Peptides. *Peptide Res.* **1994**, *7*, 195-206.

Merrifield, R.B. Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide. *J. Am. Chem. Soc.* **1963**, *85*, 2149.

Metzger, J.W.; Wiesmüller, K-H.; Gnau, V.; Brünjes, J.; Jung, G. Ion-Spray Mass Spectrometry and High-Performance Liquid Chromatography-Mass Spectrometry of Synthetic Peptide Libraries. *Angrew. Chem. Int. Ed. Engl.* **1993**, *32*, 894-896.

Meunier, J.-C.; Kouakon, Y.; Puget, A.; Moisand, C. Multiple Opiate Binding Sites in the Central Nervous System of the Rabbit: Large Predominance of a Mu Subtype in the Cerebellum and Characterization of a Kappa Subtype in the Thalamus. *Mol. Pharmacol.* **1983**, *24*, 23-29.

Meyer, J.-P.; Collins, N.; Lung, F.-D.; Davis, P.; Zalewska, T.; Porreca, F.; Yamamura, H.I.; Hruby, V.J. Design, Synthesis, and Biological Properties of Highly Potent Cyclic Dynorphin A Analogues. Analogues Cyclized between Positions 5 and 11. *J. Med. Chem.* **1994**, *37*, 3910-3917.

Millan, M.J. κ-Opioid Receptors and Analgesia. *Trends Pharmacol. Sci.* **1990**, *11*, 70-76.

Miranda, A.; Koerber, S.C.; Gulyas, J.; Lahrichi, S.L.; Craig, A.G.; Corrigan, A.; Hagler, A.; Rivier, C.; Vale, W.; Rivier, J. Conformationally Restricted Competitive Antagonists of Human/Rat Corticotropin-Releasing Factor. *J. Med. Chem.* **1994**, *37*, 1450-1459.

Mojsov, S.; Mitchell, A.R.; Merrifield, R.B. A Quantitative Evaluation of Methods for Coupling Asparagine. *J. Org. Chem.* **1980**, *45*, 555-560.

Mollereau, C.; Parmentier, M.; Mailleux, P.; Butour, J.-L.; Moisand, C.; Chalon, P.; Caput, D.; Vassart, G.; Meunier, J.-C. ORL1, A Novel Member of the Opioid Receptor Family: Cloning, Functional Expression and Localization. *FEBS Lett.* **1994**, *341*, 33-38.

Montecucchi, P.C.; De Castiglione, R.; Piani, S.; Gozzini, L.; Erspamer, V. Amino Acid Composition and Sequence of Dermorphin, a Novel Opiate-like Peptide From the Skin of *Phyllomedusa sauvagei*. *Int. J. Peptide Protein Res.* **1981**, *17*, 275-283.

Mosberg, H.I.; Hurst, R.; Hruby, V.J.; Gee, K.; Yamamura, H.I.; Galligan, J.J.; Burks, T.P. Bis-penicillamine Enkephalins Possess Highly Improved Specificity Toward δ Opioid Receptors. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5871-5874.

Müller, K.; Obrecht, D.; Knierzinger, A.; Chuck, S.; Spiegler, C.; Bannwarth, W.; Trzeciak, A.; Englert, G.; Labhardt, A.; Schönholzer, P. Building Blocks for the Induction or Fixation of Peptide Conformations. In *Perspectives in Medicinal Chemistry*, Testa, B.; Kyburz, E.; Fuhrer, W.; Giger, R., Eds.; Verlag Helvetica Acta: Basel, Switzerland, 1993, pp 515-531.

Nakanishi, S.; Inoue, A.; Kita, T.; Nakamura, M.; Chang, A.C.Y.; Cohen, S.N.; Numa, S. Nucleotides Sequence of Cloned cDNA for Bovine Corticotropin-β-lipotropin Precursor. *Nature (London)* **1979**, *278*, 423-427.

Nicolás, E.; Pedroso, E.; Giralt, E. Formation of Aspartimide Peptides in Asp-Gly Sequences. *Tetrahedron Lett.* **1989**, *30*, 497-500.

Ninkovic, M.; Hunt, S.P.; Emsom, P.C.; Iversen, L.L. The Distribution of Multiple Opiate Receptors in Bovine Brain. *Brain Res.* **1981**, *214*, 163-167.

Noda, M.; Furutani, Y.; Toyosato, M.; Hirose, T.; Inayama, S.; Nakanishi, S.; Numa, S. Cloning and Sequence Analysis of cDNA for Bovine Adrenal Preproenkephalin. *Nature (London)* **1982**, *295*, 202-206.

Nolan, W.P.; Ratcliffe, G.S.; Ree, D.C. The Synthesis of 1,6-Disubstituted Indanes which Mimic the Orientation of Amino Acid Side-Chains in a Protein Alpha-Helix Motif. *Tetrahedron Lett.* **1992**, *33*, 6879-6882.

Nomizu, M.; Inagaki, Y.; Yamashita, T.; Ohkubo, A.; Otaka, A.; Fujii, N.; Roller, P.P.; Yajima, H. Two-step Hard Acid Deprotection/cleavage Procedure for

the Solid-Phase Peptide Synthesis. *Int. J. Peptide Protein Res.* **1991**, *37*, 145-152.

Numa, S. Opioid Peptide Precursors and Their Genes. In *The Peptides Vol. 6.* Udenfriend, S.; Meienhoffer, J., Eds.; Academic Press: New York, 1984, pp 1-23.

Oka, T.; Negishi, K.; Suda, M.; Matsumiya, T.; Inazu, T.; Ueki, M. Rabbit Vas Deferens: A Specific Bioassay for Opioid κ-Receptor Agonists. *Eur. J. Pharmacol* **1980**, *73*, 235-236.

Ösapay, G.; Profit, A.; Taylor, J.W. Synthesis of Tyrocidine A: Use of Oxime Resin for Peptide Chain Assembly and Cyclization. *Tetrahedron Lett.* **1990**, *31*, 6121-6124.

Ösapay, G.; Taylor, J.W. Multiplicity Polypeptide Model Compounds. 2. Synthesis and Conformational Properties of a Highly α-Helical Uncosapeptide Constrained by Three Side-Chain to Side-Chain Lactam Bridges. *J. Am. Chem. Soc.* **1992**, *114*, 6966-6973.

Papayannopoulos, I.A.; Biemann, K. Fast Atom Bombardment and Tandem Mass Spectrometry of Synthetic Peptides and Byproducts. *Peptide Res.* **1992**, *5*, 83-90.

Pasternak, G.W.; Wood, O. Multiple Mu Opiate Receptors. *Life Sci.* **1986**, *38*, 1889-1898.

Pert, C.B.; Snyder, S.H. Opiate Receptor: Demonstration in Nervous Tissue. *Science*, **1973**, *179*, 1011-1014.

Pert, C.B.; Snyder, S.H. Opiate Receptor Binding of Agonists and Antagonist Affected Differentially by Sodium. *Mol. Pharmacol.* **1974**, *10*, 868-879.

Pfieiffer, A,; Pasi, A.; Mahraein, P.; Herz, A. A Subclassification of Kappa Sites in Human-Brain by Use of Dynorphin 1-17. *Neuropeptid*, **1981**, *2*, 89-97.

Plaué, S. Synthesis of Cyclic Peptides on Solid Support: Application to Analogs of Hemagglutinin of Influenza Virus. *Int. J. Peptide Protein Res.* **1990**, *35*, 510-517.

Rapaka, R.S.; Renugopalakrishnan, V.; Collete, T.W.; Dobbs, J.C.; Carreira, L.A.; Bhatnagar, R.S. Conformational Features of Dynorphin A(1-13): Laser Raman Spectroscopic Studies. *Int. J. Peptide Protein Res.* **1987**, *30*, 284.

Raynor, K.; Kong, H.; Chen, Y.; Yasuda, K.; Yu, L.; Bell, G.I.; Reisine, T. Pharmacological Characterization of the Cloned κ-, δ-, and μ-Opioid Receptors. *Mol. Pharmacol.* **1994**, *45*, 330-334.

Ree, D.C.; Hunter, J.C. Opioid Receptors. In *Comprehensive Medicinal Chemistry,* Sammes, P.G.; Taylor, J.B.; Emmett, J.C., Eds.; Pergamon Press: New York, 1990, pp 805-846.

Rees, D.C. Chemical Structures and Biological Activities of Non-Peptide Selective Kappa Opioid Ligands. In *Progress in Medicinal Chemistry*; Ellis, G.P.; Luscombe, D.K., Eds.; Elsevier Science Publishers B.V.: New York, 1992; Vol. 29, pp 109-139.

Renugopalakrishnan, V.; Rapaka, R.S.; Bhargava, H.N. Conformational Features of Opioid Peptides: Ligand Receptor Interactions. In *Biochemistry and Applied Physiology, Vol. IV*, Székely, J.I.; Ramabadran, K., Eds.; CRC Press: Boston, 1990, pp 54-114.

Renugopalakrishnan, V.; Rapaka, R.S.; Huang, S.G. Secondary Structural Studies of Dynorphin. In *Recent Progress in Chemistry and Biology of Centrally Acting Peptides*, Dhawan, B.N.; Rapaka, R.S., Eds.; Central Drug Research Institution: Lucknow, India, 1988, pp 71-85.

Ressler, C.; Ratzkin, H. Synthesis of β-Cyano-L-alanine and γ-Cyano-α-L-aminobutyric Acid, Dehydration Products of L-Asparagine and L-Glutamine; a New Synthesis of Amino Acid Nitriles. *J. Org. Chem.* **1961**, *26*, 3356-3360.

Rivaille, P.; Gautron, J.P.; Castro, B.; Milhaud, G. Synthesis of LH-RH Using a New Phenolic Polymer as Solid Support and "BOP" Reagent for Fragment Coupling. *Tetrahedron* **1980**, *36*, 3413-3419.

Robson, L.E.; Foote, R.W.; Maurer, R.; Kosterlitz, H.W. Opioid Binding Sites of the κ-Type in Guinea-Pig Cerebellum. *Neuroscience* **1984**, *12*, 621-627.

Schiller, P.W. Fluorescence Study on the Solution Conformation for Dynorphin in Comparison to Enkephalin. *Int. J. Peptide Protein Res.* **1983**, *21*, 307.

Schiller, P.W.; Eggiman, B.; Nguyen, T.M.-D. Comparative Structure-Function Studies with Analogs of Dynorphin-(1-13) and [Leu^2]Enkephalin. *Life Sci.* **1982**, *31*, 1777-1780.

Schiller, P.W.; Nguyen, T.M.-D.; Lemieux, C. Synthesis and Opioid Activity Profile of Cyclic Dynorphin Analogs. *Tetrahedron* **1988**, *44*, 733-743.

Schiller, P.W.; Nguyen, T.M.-D; Maziak, L.A. Synthesis and Activity Profiles of Novel Cyclic Opioid Peptide Monomers and Dimers. *J. Med. Chem.* **1985a**, *28*, 1766-1771.

Schiller, P.W.; Nguyen, T.M.-D.; Miller, J. Synthesis of Side-Chain to Side-Chain Cyclized Peptide Analogs on Solid Supports. *Int. J. Peptide Protein Res.* **1985b**, *25*, 171-177.

Schmidt, R.; Neubert, K. Cyclization Studies with Tetra- and Pentapeptide Sequences Corresponding to β-Casomorphins. *Int. J. Peptide Protein Res.* **1991**, *37*, 502-507.

Schwyzer, R. Estimated Conformation, Orientation and Accumulation of Dynorphin A-(1-13)-tridecapeptide on the Surface of the Neutral Lipid Membranes. *Biochemistry* **1986a**, *25*, 4281-4286.

Schwyzer, R. Molecular Mechanism of Opioid Receptor Selection. *Biochemistry* **1986b**, 25, 6335-6342.

Schwyzer, R. Peptide-Membrane Interactions and a New Principle in Quantitative Structure-Activity Relationships. *Biopolymers* **1991**, *31*, 785-792.

Schwyzer, R. How Do Peptides Interact with Lipid Membranes and How Does This Affect Their Biological Activity? *Brazillian J. Med. Biol. Res.* **1992a**, *25*, 1077-1089.

Schwyzer, R. Conformations and Orientations of Amphiphilic Peptides Induced by Artificial Lipid Membranes: Correlations with Biological Activity. *Biochem. Mol. Biol.* **1992b**, *3*, 347-379.

Schwyzer, R. 100 Years Lock-and-Key Concept: Are Peptide Keys Shaped and Guided to Their Receptors by the Target Cell Membrane? *Biopolymers* **1995**, *37*, 5-16.

Schwyzer, R.; Sieber, P. Die Synthese von Gramicidin S. *Helv. Chim. Acta* **1957**, *40*, 624-639.

Shearman, G.T.; Schulz, R.; Schiller, P.W.; Herz, A. Generalization Tests with Intraventricularly Applied Pro-enkephalin B Derived Peptides in Rats Trained to Discriminate the Opioid Kappa Receptor Agonist Ethylketocyclazocine. *Psychopharmacology* **1985**, *85*, 440-443.

Shoemaker, K.R.; Kim, P.S.; York, E.J.; Stewart, J.M.; Baldwin, R.L. Tests of the Helix Dipole Model for Stabilization of α-Helices. *Nature (London)* **1987**, *326*, 563-567.

Simon, E.J. Opiate Receptor Binding in Drug Reserach. In *Clinical Pharmacology, Vol. 5*, O'Brien, R.A., Ed.; Marcel Dekker, Inc.: New York, 1986a, pp 183-202.

Simon, E.J. Progress in the Characterization of the Opioid Receptor Subtypes: Peptides as Probes. Future Directions. *Natl. Inst. Drug Abuse Res. Monogr.* **1986b**, *70*, 155-174.

Simon, E.J. Opioid Receptors and Endogenous Opioid Peptides. *Med. Res. Rev.* **1991**, *11*, 357-374.

Simon, E.J.; Hiller, J.M. Opioid Peptides and Opioid Receptors. In *Basic Neurochemistry: Molecular, Cellular, and Medical Aspects*, 5th ed.; Siegel, G.J.et al. Eds.; Raven Press, Ltd., New York: 1994, pp 321-339.

Simon, E.J.; Hiller, J.M.; Edelman, I. Stereospecific Binding of the Potent Narcotic Analgesic ^{3}H-Etorphine to Rat Brain Homogenate. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 1947-1949.

Simon, E.J.; Hiller, J.M.; Groth, J.; Itzhak, Y.; Holland, M.J.; Beck, S.G. The Nature of Opiate Receptors in Toad Brain. *Life Sci.* **1982**, *31*, 1367-1370.

Snyder, K.R.; Murray, T.F.; DeLander, G.E.; Aldrich, J.V. Synthesis and Opioid Activity ofDynorphin A-(1-13)NH_2 Analogues Containing *cis*- and *trans*-4-Aminocyclohexanecarboxylic Acid. *J. Med. Chem.* **1993**, *36*, 1100-1103.

Snyder, K.R.; Story, S.C.; Heidt, M.E.; Murray, T.F.; DeLander, G.E.; Aldrich, J.V. Effect of Modification of the Basic Residues of Dynorphin A-(1-13) Amide on Kappa Opioid Receptor Selectivity and Opioid Activity. *J. Med. Chem.* **1992**, *35*, 4330-4333.

Stewart, J.M.; Young, J.D. The Chemistry of Solid Phase Peptide Synthesis. In *Solid Phase Synthesis*. 2rd ed., Pierce Chemical Company: Illinois, 1984, pp 1-52.

Story, S.C.; Aldrich, J.V. Side-Product Formation during Cyclization with HBTU on a Solid Support. *Int. J. Peptide Protein Res.* **1994**, *43*, 292-296.

Story, S.C.; Murray, T.F.; DeLander, G.E.; Aldrich, J.V. Synthesis and Opioid Activity of 2-Substituted Dynorphin A-(1-13) Amide Analogues. *Int. J. Pept. Protein Res.* **1992**, *40*, 89-96.

Takemori, A.E.; Larson, D.L.; Portoghese, P.S. The Irreversible Narcotic Antagonistic and Reversible Agonistic Properties of the Fumaramate Methyl Ester Derivative of Naltrexone. *Eur. J. Pharmacol.* **1981**, *70*, 445-451.

Takemori, A.E.; Portoghese, P.S. Affinity Labels for Opioid Receptors. *Ann. Rev. Pharmacol. Toxicol.* **1985**, *25*, 193-223.

Tam, J.P.; Heath, W.F.; Merrifield, R.B. S_N^2 Deprotection of Synthetic Peptides with a Low Concentration of HF in Dimethyl Sulfide: Evidence and Application in Peptide Synthesis. *J. Am. Chem. Soc.* **1983**, *105*, 6442-6455.

Tam, J.P.; Merrifield, R.B. Strong Acid Deprotection of Synthetic Peptides: Mechanism and Methods. In *The Peptides, Vol. 9*, Udenfriend, S.; Meienhofer, J., Eds.; Academic Press: New York, 1987, pp 185-248.

Tam, J.P.; Riemen, M.W.; Merrifield, R.B. Mechanism of Aspartimide Formation: The Effects of Protecting Groups, Acid, Base, Temperature and Time. *Peptide Res.* **1988**, *1*, 6-18.

Taylor, J.W. Peptide Models of Dynorphin A(1-17) Incorporating Minimally Homologous Substitutes for the Potential Amphiphilic β Strand in Residues 7-15. *Biochemistry* **1990**, *29*, 5364-5373.

Terenius, L. Stereospecific Interaction between Narcotic Analgesics and a Synaptic Plasma Membrane Fraction of Rat Cerebral Cortex. *Acta. Pharmacol. Toxicol. Copen.* **1973**, *32*, 317-320.

Toniolo, C.; Crisma, M.; Formaggio, F.; Caruicchioni, G. Structures of Peptides from α-Amino Acids Methylated at the C^{α}-Atom. *Biopolymers* **1993**, *33*, 1061-1071.

Traynor, J.R.; Elliott, J. δ-Opioid Receptor Subtypes and Cross-Talk with μ-Receptors. *Trends Pharmacol. Sci.* **1993**, *14*, 84-86.

Turcotte, A.; Lalonde, J.-M.; St.-Pierre, S.; Lemaire, S. Dynorphin-(1-13) I. Structure-Function Relationships of Ala-Containing Analogs. *Int. J. Peptide Protein Res.* **1984**, *23*, 361-367.

Uhl, G.R.; Childers, S.; Pasternak, G. An Opiate-Receptor Gene Family Reunion. *Trends Neurosci.* **1994**, *17*, 89-93.

Valette, A.; Reme, J.M.; Pontonnier, G.; Cros, J. Specific Binding for Opiate-like Drugs in the Placenta. *Biochem. Pharmacol.* **1980**, *29*, 2657-2661.

van Gorkom, L.C.M.; Lancaster, C.R.D.; St-Pierre, S.; Bothner-By, A.A.; Epand, R.M. The Membrane Mediated Conformation of Dynorphin A(1-13) as Studied by Transferred Nuclear Overhauser Effect Spectroscopy. In *Peptides: Proceedings of the 12th American Peptide Symposium*; Smith, J.A.; Rivier, J.E., Eds.; ESCOM; Leiden; **1992**, pp 233-234.

Vaughn, J.B.Jr.; Taylor, J.W. Proton NMR and CD Solution Conformation Determination and Opioid Receptor Binding Studies of a Dynorphin A(1-17) Model Peptide. *Biochim. Biophys. Acta* **1989**, *999*, 135-146.

Vestal, M.L. Liquid Chromatography-Mass Spectrometry. *Meth. Enzym.* **1990**, *193*, 107-130.

Wade, J.D.; Bedford, J.; Sheppard, R.C.; Tregear, G.W. DBU as an N^{α}-Deprotecting Reagent for the Fluorenylmethoxycarbonyl Group in Continueous Flow Solid-Phase Peptide Synthesis. *Peptide Res.* **1991**, *4*, 194-199.

Waki, M.; Kitajma, Y.; Izumiya, N.; A Facile Synthesis of N^2-Protected L-2,3-Diaminopropanoic Acid. *Synthesis* **1981**, 266-268.

Weiner, P.K.; Kollman, P.A. AMBER: Assisted Model Building with Energy Refinement. A General Program for Modeling Molecules and Their Interactions. *J. Comput. Chem.* **1981**, *2*, 287-303.

Weiner, S.J., Kollman, P.A. An All Atom Force Field for Simulations of Proteins and Nucleic Acids. *J. Comput. Chem.* **1986**, *7*, 230-252.

Weiner, S.J.; Kollman, P.A.; Case, D.A.; Singh, U.C.; Ghio, C.; Alagona, G.; Profeta, S., Jr.; Weiner, P.K. A New Force Field for Molecular Mechanical Simulation of Nucleic Acids and Proteins. *J. Am. Chem. Soc.* **1984**, *106*, 765-784.

Weitz, C.J.; Lowney, L.I.; Faull, K.F.; Feistner, G.; Goldstein, A. Morphine and Codeine from Mammalian Brain. *Proc. Natl. Acad. Sci.* **1986**, *83*, 9784-9788.

Wollemann, M.; Benyhe, S.; Simon, J. The Kappa-Opioid Receptor: Evidence for the Different Subtypes. *Life Sci.* **1993**, *52*, 599-611.

Xie, G.-X.; Meng, F.; Mansour, A.; Thompson, R.; Hoversten, M.T.; Goldstein, A.; Watson, S.J.; Akil, H. Primary Structure and Functional Expression of a Guinea Pig κ Opioid (Dynorphin) Receptor. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 3779-3783.

Xue, C.-B.; Naider, F. Application of N-(*tert*-Butyloxycarbonyl)amino Acid N-Carboxyanhydrides in Solid-Phase Peptide Synthesis. *J. Org. Chem.* **1993**, *58*, 350-355.

Yasuda, K.; Raynor, K.; Kong, H.; Breder, C.; Takeda, J.; Reisine, T.; Bell, G. Cloning and Functional Comparison of Kappa and Delta Opioid Receptors from Mouse Brain. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6736-6740.

Zastawny, R.L.; George, S.R.; Nguyen, T.; Cheng, R.; Tsatsos, J.; Briones-Urbina, R.; O'Dowd, B.F. Cloning, Characterization, and Distribution of a μ-Opioid Receptor in Rat Brain. *J. Neurochem.* **1994**, *62, 2099-2105.*

Zimmer, S.; Hoffmann, E.; Jung, G.; Kessler, H. "Head-to-Tail" Cyclization of Heaxapeptides Using Different Coupling Reagents. *Liebigs Ann. Chem.* **1993**, 497-501.

Zukin, R.S.; Eghbali, M.; Olive, D.; Unterwald, E.M.; Tempel, A. Characterization and Visualization of Rat and Guinea Pig Brain Kappa Opioid Receptors: Evidence for Kappa 1 and Kappa 2 Opioid Receptors. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 4061-4065.

Zukin, R.S.; Zukin, S.R. Demonstration of [^{3}H]Cyclazocine Binding to Multiple Opiate Receptor Sites. *Mol. Pharmacol.* **1981**, *20*, 246-254.

APPENDICES

APPENDIX A

Nomenclature for Peptide Fragment Ions (Positive Ions)

N-terminal Ions

a_n: $H-(NH-CHR-CO)_{n-1}-\overset{+}{N}H=CHR_n$

or

$$H-\overset{H^+}{\overbrace{(NH-CHR-CO)_{n-1}}}-NH-\underset{\|}{\overset{CR_n{}^aR_n{}^b}{CH}}$$

$a_n + 1$: $H-\overset{H^+}{\overbrace{(NH-CHR-CO)_{n-1}}}-NH-\underset{|}{\overset{R_n}{CH}}\cdot$

b_n: $H-(NH-CHR-CO)_{n-1}-NH-CHR_n-C\equiv O^+$

c_n: $H-\overset{H^+}{\overbrace{(NH-CHR-CO)_n}}-NH_2$

d_n: $H-\overset{H^+}{\overbrace{(NH-CHR-CO)_{n-1}}}-NH-\underset{\|}{\overset{CR_n{}^b}{CH}}$

C-terminal Ion Types

v_n: $HN=CH-CO-\overset{H^+}{\overbrace{(NH-CHR-CO)_{n-1}}}-OH$

w_n: $\underset{\|}{\overset{CR_n{}^b}{CH}}-CO-\overset{H^+}{\overbrace{(NH-CHR-CO)_{n-1}}}-OH$

x_n: $^+O\equiv C-NH-CHR_n-CO-(NH-CHR-\widetilde{CO})_{n-1}-OH$

or

$$O=C=N-\overset{H^+}{\overbrace{CHR_n-CO-(NH-CHR-CO)_{n-1}}}-OH$$

y_n: $H-\overset{H^+}{\overbrace{(NH-CHR-CO)_n}}-OH$

$y_n - 2$: $\overset{H^+}{\overbrace{HN=CR_n-CO-(NH-CHR-CO)_{n-1}}}-OH$

z_n: $\underset{\|}{\overset{CR_n{}^aR_n{}^b}{CH}}-CO-\overset{H^+}{\overbrace{(NH-CHR-CO)_{n-1}}}-OH$

$z_n + 1$: $CHR_n-CO-\overset{H^+}{\overbrace{(NH-CHR-CO)_{n-1}}}-OH$

Non-Sequence Specific Ions

Internal acyl ions (denoted by single letter codes) (e.g., GA at *m/z* 129 for R = H, CH_3):

$$H_2N—CHR—CO—NH—CHR—C{\equiv}O^+$$

Internal immonium ions (denoted by single-letter code followed by the notation −28):

$$H_2N—CHR—CO—\overset{+}{N}H{=}CHR$$

Amino acid immonium ions (denoted by single letter code) (e.g., F at *m/z* 120 for R = $C_6H_5CH_2$):

$$\overset{+}{H_2N}{=}CHR$$

Loss of amino acid side chains from $(M + H)^+$ is denoted as the single letter code of the amino acid involved, preceded by a minus sign (e.g., −V for $[M + H - 43]^+$).

* R represents the side chains of the amino acids; R_n^a and R_n^b are the beta substituents of the *n*th amino acid.

Source: Biemann, K. *Meth. Ensym.* **1990**, *193*, 886-887.

APPENDIX B

Elemental Analysis of FmocDap(Boc) · 1 H_2O

ELEMENT	THEORETICAL (%)	FOUND (%)
C	62.13	62.08
H	6.35	6.33
N	6.31	6.40