AN ABSTRACT OF THE THESIS OF

Sandra Catherine Story for the degree of Doctor of Philosophy in Pharmacy presented on May 2, 1990.

Title: Solid Phase Synthesis of Dynorphin Analogues as Probes of Kappa Opioid Receptors.

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Abstract approved: _____
Jane V. Aldrich, Ph.D.

The first part of this project consisted of a series of 2-substituted dynorphin A-(1-13) amide analogues which were synthesized by solid phase synthesis using the Boc chemical protocol. Following HF cleavage and purification, the peptides were evaluated for opioid activity in the electrically stimulated guinea pig ileum and for kappa opioid receptor binding using a radioligand binding assay. Amino acid substitution at the 2 position of dynorphin A-(1-13) amide produced marked differences in both opioid activity and kappa receptor binding. The IC_{50} values for the analogues varied over four orders of magnitude in both pharmacological assays.

The parent compound, dynorphin A-(1-13) amide, was the most potent compound tested. The [D-Asn^2]-, [D-Ser^2]-, and [D-Met^2]dynorphin A-(1-13) amide analogues were the most potent of the synthetic peptides prepared

while the [L-Lys^2]- and [D-Lys^2] dynorphin A-(1-13) amide were the least potent. This data suggest that a basic residue in the "message" sequence of the peptide is not well tolerated and that an α-helical conformation is probably not necessary for opioid activity and receptor binding.

The second part of this project involved novel solid phase synthetic methodology to prepare a protected peptide fragment which could be further modified to incorporate a reactive functionality into the peptide. Three different resins were analyzed in an effort to prepare a model protected peptide amide using the Fmoc chemical protocol.

The polyamide resin was found to be incompatible with our batch-type instrument. The resin transformed into a solid gelatinous mass that resulted in a very poor recovery of resin after synthesis (<30% expected). The "Rink" polystyrene resin provided an excellent medium for automated peptide synthesis with an expected weight gain of >99%. The flaw with this resin was its inability to release the protected peptide upon cleavage (<10% recovery). Data from amino acid analysis indicated that the peptide was present on the resin but not liberated upon cleavage.

A modified polystyrene resin designed and synthesized in this project prevailed as the only feasible support for the adequate production of a protected peptide fragment. The synthesis was compatible with the batch instrument (>70% expected weight gain) and the protected peptide could be cleaved from the resin (>50% recovery). This polystyrene resin could have wide ranging flexibility for use in the preparation of protected peptide amides.

Solid Phase Synthesis of Dynorphin Analogues as Probes of Kappa Opioid Receptors

by

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The completion of this degree represents the highest mountain I've ever had to climb. The price to reach the top, both personally and professionally, was higher than I ever imagined I'd have to pay.

"Gonna break these chains around me
Gonna learn to fly again
May be hard, But I'll do it
When I'm back on my feet again

Soon all these tears will be dryin'
Soon these eyes will see the sun
Might take time, But I'll see it
When I'm back on my feet again.

When I'm back on my feet again
I'll walk proud down this street again
And they'll all look at me again
And they'll see that I'm strong

And I'm not gonna crawl again
I will learn to stand tall again
No, I'm not gonna fall again
I'll be back on my feet again."

-- Adapted from D. Warren

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Non-standard Abbreviations

AA	amino acid
AcCN	acetonitrile
Boc	*tert*-butyloxycarbonyl
Bzl	benzyl
Clz	2-chlorobenzyloxycarbonyl
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DCU	dicyclohexylurea
DIPEA	diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DVB	1,4-divinylbenzene
EtOAc	ethyl acetate
EtOH	ethanol
FDNB	2,4-dinitrofluorobenzene
Fmoc	9-fluorenylmethoxycarbonyl
HOAc	acetic acid
HOBt	1-hydroxybenzotriazole
iPrOH	2-propanol
MBHA	4-methylbenzhydrylamine
MeOH	methanol
Mtr	4-methoxy-2,3,6-trimethylbenzenesulfonyl
OBt	1-benzotriazolyl ester
OBzl	benzyl ester
OMe	methyl ester
OPfp	pentafluorophenyl ester
OtBu	*tert*-butyl ester
Pam	phenylacetamidomethyl
Pmc	2,2,5,7,8-pentamethylchroman-6-sulphonyl
SPPS	solid phase peptide synthesis
SPS	solid phase synthesis
tBu	*tert*-butyl
TFA	trifluoroacetic acid
TNBSA	2,4,6-trinitrobenzenesulfonic acid
Tos	4-toluenesulfonyl (tosyl)
Z	benzyloxycarbonyl

SOLID PHASE SYNTHESIS OF DYNORPHIN ANALOGUES AS PROBES OF KAPPA OPIOID RECEPTORS

CHAPTER 1

INTRODUCTION

The analgesic activity of opiates has been known for thousands of years. The first undisputed written reference to the medicinal use of poppy juice came from Theophrastus in the third century B.C. (Jaffe and Martin, 1985). Morphine, named after Morpheus the Greek god of dreams, is a product of the poppy, *Papaver somniferum* (Evans et al., 1988). Serturner discovered the analgesic effects of morphine in 1806 and shortly thereafter the undesirable addiction liability (Rapaka, 1986). The problem of addiction associated with opioid analgesics inspired scientists to search for compounds that would be potent analgesics but not have the same potential to produce addiction.

It has only been within the last fifteen years that the endogenous opioid peptides were isolated, identified and associated with analgesic activity. The pharmacological effects of opioid peptides are quite complex. It is thought that analgesia is a result of the interaction of the endogenous peptide with the opioid receptors in the brain and spinal cord.

Dynorphin is an extremely potent endogenous opioid peptide. To indicate the potency of this peptide its name was derived from the Greek word *dynamis*, meaning power (Goldstein et al., 1979). Dynorphin preferentially interacts with kappa opioid receptors, which are present in high concentrations in the spinal cord (Goldstein, 1984). The analgesia associated with pregnancy has been connected to dynorphin and kappa receptors in the spinal cord (Sander and co-workers; 1988, 1989). Further, kappa receptors have been found in human placental tissue (Agbas et al., 1988). One suggestion is that analgesic drugs that preferentially interact at kappa opioid receptors may have the potential for a lower addiction liability than morphine and its derivatives.

A current goal in analgesic research is to separate the desired analgesic effect from undesired effects such as addiction. Separate receptor types may have different roles in producing both desirable and undesirable effects associated with narcotic analgesics such as morphine and codeine. Information on how opioid peptides interact with their receptors could lead to the development of a non-addictive, peptidomimetic analgesic or an improved drug for the treatment of addiction.

This thesis project was divided into two parts in attempts to define further the physiological functions of the kappa opioid receptors. The first part consisted of a series of 2-substituted dynorphin A-(1-13) amide analogues, which were synthesized and evaluated for opioid activity in the electrically

stimulated guinea pig ileum and for kappa receptor binding using a radioligand binding assay.

The second part was directed toward the synthesis of affinity labels derived from dynorphin. It involved novel chemistry to prepare a protected peptide amide fragment of dynorphin A-(1-13) amide by solid phase synthesis. This fragment could be used as an intermediate for additional modification to incorporate a reactive functionality into the peptide. This portion of the project involved evaluating three different solid supports in an attempt to produce a protected peptide amide.

PART ONE

2-SUBSTITUTED DYNORPHIN A-(1-13) AMIDE ANALOGUES

CHAPTER 2

LITERATURE REVIEW

2.1 Multiple Opioid Receptors

Opioid receptors are bound tightly to cell membranes indicating that the binding sites are integral membrane proteins. The glycoprotein nature of the opioid receptors is consistent with the properties of integral membrane proteins (Simon and Hiller, 1984).

The existence of multiple opioid receptors was first suggested in the mid-sixties (Portoghese, 1965; Martin, 1967). A decade later Martin proposed three types of opioid receptors and named them according to the ligand used in the study. The three opioid receptors he suggested were: the mu (μ) receptor, the kappa (κ) receptor and the sigma (σ) receptor. The mu receptor was named for morphine; kappa for ketocyclazocine; and sigma for SKF 10,047 (N-allyl normetazocine) (Martin et al., 1976). The δ receptor was later proposed and named after the mouse vas deferens used in the identifying assay (Hughes et al., 1975); DADLE ([D-Ala^2,D-Leu^5]enkephalin) is an

agonist used to study the delta receptor (Lord et al., 1977). Later evidence has supported the multiple opioid receptor hypothesis (see Paterson et al., 1983).

Opioid receptors have been found in the central nervous system (CNS) and in some peripheral tissues such as the pituitary and adrenal glands, intestinal tract and vas deferens. Opioid receptors have been found in all vertebrates, including man, and have recently found in some invertebrates. The distribution of the opioid receptors in the CNS include the limbic system and all areas associated with pain perception and modulation (Simon & Hiller, 1984).

The tissues used in characterizing the opioid receptors vary in the receptors they contain (Figure 2.1). The guinea pig ileum has both mu and kappa receptors, but the two receptors can be distinguished by their differing affinities for the classical opioid antagonist naloxone (Goldstein, 1983). The mouse vas deferens contains mu, kappa and delta receptors; the hamster vas deferens contains only delta receptors (McKnight et al. 1984); whereas the rabbit vas deferens contains only kappa receptors (Oka et al., 1984).

The whole guinea pig brain has approximately 30% kappa receptor binding sites while the cerebellum has greater than 80% κ binding sites (Robson et al., 1984). The rat brain contains mostly μ and δ binding sites with only about 12% of κ receptors (Goldstein, 1984).

Opioid Receptor	Classical Agonist	Proposed Endogenous Ligand
μ	morphine	uncertain
δ	DADLE	enkephalins
κ	ketocyclazocine	dynorphin

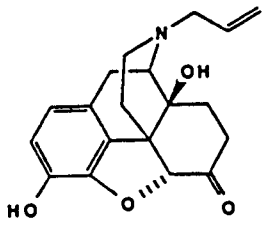
Figure 2.1 Opioid Receptors and Agonists

2.1.1 Kappa Receptor

The kappa opioid receptor was one of the multiple receptor types described by Martin (Martin, 1967). The study by Woods with bremazocine supported the existence of the kappa opioid receptor Martin proposed (Woods et al., 1979). By using guinea pig brain, which is rich in kappa receptors, in combination with blockers for the mu and delta receptors, Kosterlitz's group demonstrated the existence of a separate kappa site (Magnan et al., 1982). Additional experimental evidence for the existence of kappa receptors came from studies using the irreversible opioid antagonists, β-chlornaltrexamine (CNA) (Figure 2.2), a nonselective ligand (James et al., 1982), and naltrexone fumaramate methyl ester (β-FNA) (Figure 2.2), a μ-selective ligand (Huidobro-Toro et al., 1982). Dynorphin selectively protected the kappa receptors from alkylation by CNA while the mu and delta receptors were destroyed (James et al., 1982). β-FNA failed to inhibit the potency of

either dynorphin or EKC, nor did it cause a significant increase in the apparent naltrexone K_e value against dynorphin or EKC (Huidobro-Toro et al., 1982).

Kappa receptors are distributed throughout the CNS. In the human brain, most regions were found to have a high proportion of kappa receptors. The highest levels of kappa receptors in the human brain were found in the hypothalamus (Itzhak et al., 1982). Kappa opioid receptors have been shown to be involved in mediating analgesia in the spinal cord (Höllt, 1986).



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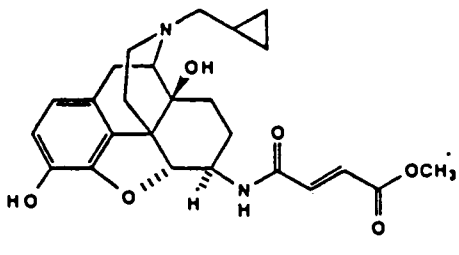
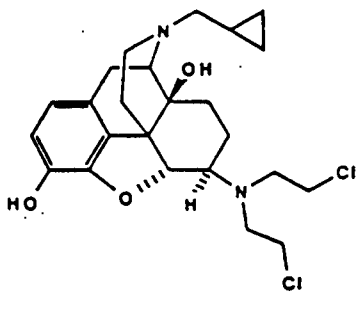


β -FNA



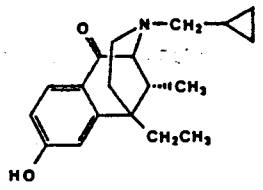
β -CNA

Figure 2.2 μ and Nonselective Antagonists Used to Characterize Kappa Receptors

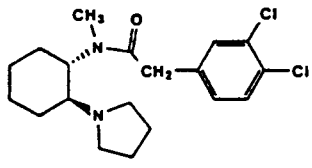
Source: Zimmerman, D.; Leander, J. *J. Med. Chem.* **1990**, *33*, p 898.

2.1.1.1 Kappa Agonists

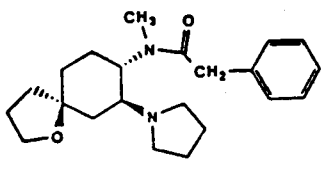
Ketocyclazocine and ethylketocyclacine (EKC) (see Figure 2.3) were first used to characterize kappa receptors and are considered to be nonselective kappa agonists (Zimmerman and Leander, 1990). The second generation of kappa agonists included bremazocine (Romer et al., 1980), tifluadom (Romer et al., 1982) and Mr 2034 (Ensinger, 1983) which were used to study kappa receptor pharmacology. Unfortunately, these ligands show considerable cross reactivity at the mu receptor (Goldstein, 1984). The current generation of kappa agonists is based on U-50,488 (Szmuszkovicz and Von Voigtlander, 1982), which is a potent novel opiate more selective for the kappa opioid receptor (Goldstein, 1984). Modifications of U-50,488 have led to more selective kappa agonists including U-62,066 (Peters et al., 1987), U-69,593 (Lahti et al., 1985), PD117302 (Leighton et al., 1987) and ICI199441 (Costello et al., 1988) (see Figure 2.3). Radioligand binding assays are currently being performed using a tritiated analogue of U-69,593 (Lahti et al., 1985).



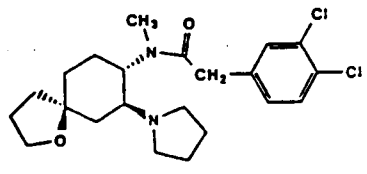
ETHYLKETOCYCLAZOCINE



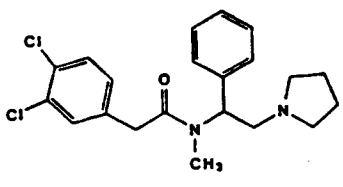
U-50,488



U-69,593



U-62,066



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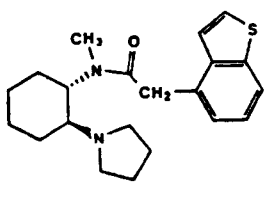


PD117302

Figure 2.3 Kappa Agonists

Source: Zimmerman, D.; Leander, J. *J. Med. Chem.* **1990**, *33*, pp 896-897.

2.1.1.2 Kappa Antagonists

Kappa receptor antagonists include Mr 2266 (Merz et al., 1975) and Win 44,441-3 (Michne et al., 1978), which have benzomorphan structures, but they also have poor kappa receptor selectivity (Kosterlitz et al., 1981; Ward and Takemori, 1983). The first selective kappa antagonists (see Figure 2.4) were TENA (Portoghese and Takemori, 1985), binaltorphimine and nor-binaltorphimine (Portoghese et al., 1987a,b). Binaltorphimine, nor-binaltorphimine and TENA are ligands that have a spacer connecting two naltrexone-like molecules (Portoghese et al., 1988). Experiments suggested that only one of the naltrexamine molecules was required for kappa receptor selectivity (Portoghese et al., 1988). The first site-directed kappa receptor inhibitors reported were compound 1 (deCosta, et al., 1989a) and UPHIT (deCosta, et al., 1989b). UPHIT will acylate κ receptors following *in vivo* administration. However, compound 1 and UPHIT do not bind receptors previously thought to be kappa receptors (deCosta, et al., 1989a). These data could support the theory of kappa receptor subtypes.

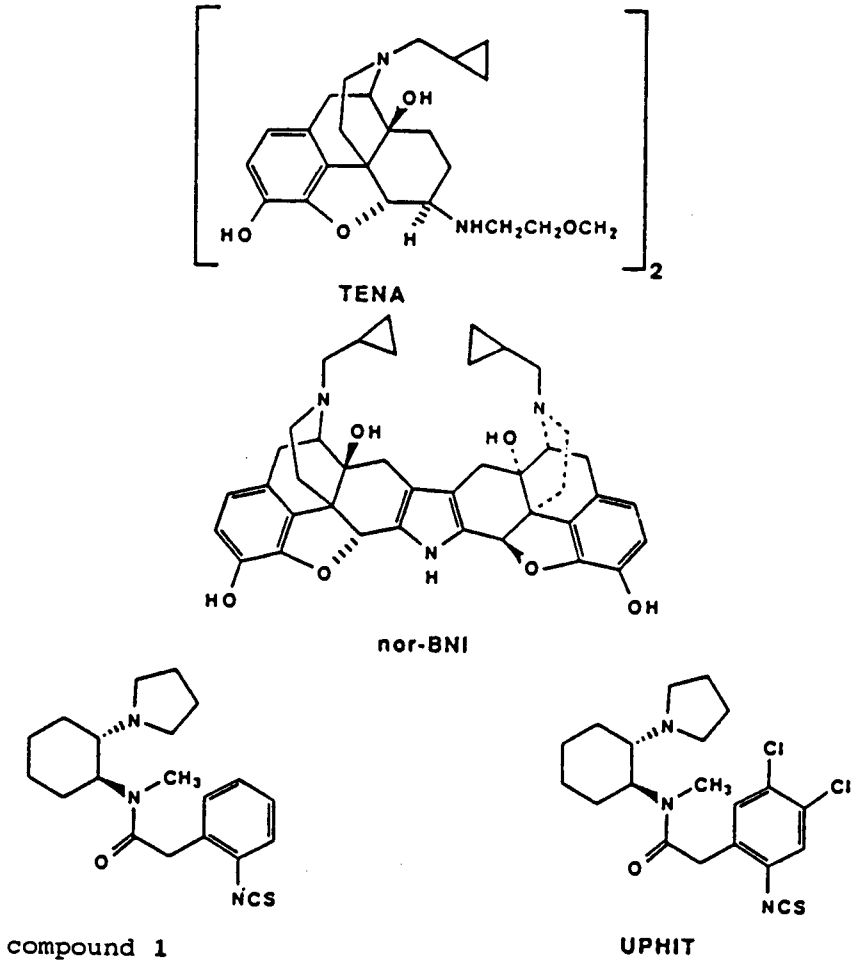


Figure 2.4 Kappa Antagonists

Source: Zimmerman, D.; Leander, J. *J. Med. Chem.* **1990**, *33*, p 899.

2.1.2 Kappa Receptor Subtypes

The question concerning the existence of kappa receptor subtypes is a complex area that has been highly debated. Recently, it was proposed that there may be as many as four kappa receptor subtypes (Clark et al., 1989). The first evidence for the existence of kappa receptor subtypes came from CNS binding studies (Attali et al., 1981; Pfeiffer et al., 1981) and cross tolerance experiments in smooth muscle preparations (Wuster and co-workers, 1981a,b). Two kappa receptor subtypes were proposed based on studies in the guinea pig spinal cord. The guinea pig spinal cord appears to be essentially without either μ or δ receptors (Gouarderes et al., 1981). The two κ subtypes proposed were: (1) the κ_1 site, that is insensitive to DADLE ([D-Ala^2,D-Leu^5]enkephalin) and corresponds to the previously described kappa receptors in the guinea pig cerebellum (Kosterlitz et al., 1981); and (2) κ_2, which is sensitive to DADLE and corresponds to the benzomorphan site characterized in the rat brain (Chang et al., 1981) and spinal cord (Gouarderes et al., 1982). Dynorphin A-(1-17) binds better to κ_1 than κ_2, but the predominant receptor subtype in the human spinal cord was proposed to be the κ_2 receptor (Attali et al., 1982 a,b).

It has recently been proposed to reclassify the κ_1 receptor sites into subclasses consisting of κ_{1a} and κ_{1b} (Clark et al., 1989). Both of these κ_1 receptor subtypes are sensitive to U-50,488. Dynorphin A labels both of these receptor subtypes, but dynorphin B (Fischli, 1982a,b; Goldstein, 1983) and α-

neoendorphin (Kangawa and co-workers, 1979, 1981) are more sensitive to the κ_{1b} receptor site (Clark et al., 1989).

A fourth kappa receptor subtype, κ_3, was proposed from work using the bovine adrenal medulla (Castanas et al., 1985a,b). This is a high affinity site which is DADLE insensitive and apparently absent from the guinea pig spinal cord (Attali et al., 1982a,b). This receptor subtype is insensitive to U-50,488 (Price et al., 1989) and has been further characterized using the mixed agonist/antagonist, NalBzOH (6-desoxy-6-benzoylhydrazido-N-allyl-14-hydroxydihydronomorphinone) (Gistrak et al., 1989). There was no analgesic cross-tolerance between NalBzOH and either morphine or U-50,488, implying a selective κ_3 mechanism of action (Gistrak et al., 1989).

2.2 Endogenous Opioid Peptides

The enkephalins, leucine enkephalin (Leu-enkephalin) and methionine enkephalin (Met-enkephalin) are the smallest endogenous opioid peptides (see Figure 2.5) and were the first endogenous opioid peptides discovered (Hughes et al., 1975). They consist of five amino acid residues differing only by the C-terminal amino acid. α- and β- neoendorphin are medium sized extended Leu-enkephalin peptides. The dynorphin family, dynorphin A and dynorphin B, contains larger extended leucine-enkephalins. β-Endorphin, the largest opioid peptide, is an extended methionine-enkephalin derivative. The

binding affinity for opioid peptides is generally in the nanomolar range (Simon & Hiller, 1984).

The endogenous ligand at the mu receptor is still uncertain despite considerable research (Rapaka, 1986) (see Figure 2.1). The endogenous ligand at the kappa receptor is thought to be dynorphin (Chavkin et al., 1982), while the enkephalins are endogenous ligands at the delta receptor (Hughes et al., 1975).

Dynorphin A[Dynorphin, Dynorphin-(1–17)]	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH
Dynorphin A-(1–9)	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-OH
Dynorphin B (Rimorphin)	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr-OH
Dynorphin B-(29) (Leumorphin, Rimorphin-(29)	H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr-Arg-Ser-Gln-Glu-Asp-Pro-Asn-Ala-Tyr-Tyr-Glu-Glu-Leu-Phe-Asp-Val-OH
β_h-Endorphin	H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu-OH
[Leu]enkephalin	H-Tyr-Gly-Gly-Phe-Leu-OH
Leumorphin, *see* Dynorphin B-29	
[Met]enkephalin	H-Tyr-Gly-Gly-Phe-Met-OH
α-Neoendorphin	H-Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys-OH
β-Neoendorphin	H-Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-OH
Prodynorphin [Proenkephalin B (mature precursor protein)]	
Proenkephalin [Proenkephalin A (mature precursor protein)]	
Rimorphin, *see* Dynorphin B	
Rimorphin-29, *see* Dynorphin B-29	

Figure 2.5 Structure of Endogenous Opioid Peptides

Source: Udenfried, S.; Meienhofer, J.; Eds. *The Peptides: Analysis, Synthesis, Biology*; Academic: Orlando, 1984; Vol. 6, pp xix-xxx.

2.3 Precursor Proteins

Three precursor genes code for all currently known mammalian opioid peptides (Figure 2.6). The three precursor proteins are proopiomelanocortin (POMC), proenkephalin, and prodynorphin (see Höllt, 1986; and Cox, 1982).

POMC is a 31 kilodalton glycoprotein that is the precursor of adrenocorticotropic hormone (ACTH), β-lipotropin, β-endorphin and γ-melanocyte-stimulating hormone (γ-MSH) (Evans et al., 1988). Peptides derived from POMC have a narrow distribution of products in the brain compared with the other two precursor genes (Evans et al., 1988). The highest concentration of mammalian POMC products has been found in the pituitary (Evans et al., 1988).

Proenkephalin is similar in molecular weight to POMC but it is unique because it contains multiple copies of the enkephalin sequence. Peptides derived from proenkephalin are Met-enkephalin, Met-enkephalin-Arg^6-Phe^7, Met-enkephalin-Arg^6-Gly^7-Leu^8 and Leu-enkephalin (Höllt, 1986). The peptides produced from proenkephalin have a much wider distribution than those derived from POMC. The peptide products are found throughout the central and peripheral nervous system. The highest concentration of mammalian proenkephalin peptides is found in the adrenal glands (Evans et al., 1988).

Products from prodynorphin include α-neoendorphin, β-neoendorphin, dynorphin A-(1-17), dynorphin-(1-32), dynorphin B and leumorphin (Evans et al., 1988). Prodynorphin is similar in size to both POMC and proenkephalin. All opioid peptides derived from prodynorphin, except Leu-enkephalin, have shown selectivity for kappa opioid receptors with dynorphin A-(1-17) being the most potent. The substantia nigra in both rat and humans has one of the highest concentrations of dynorphins within the CNS (Höllt, 1986).

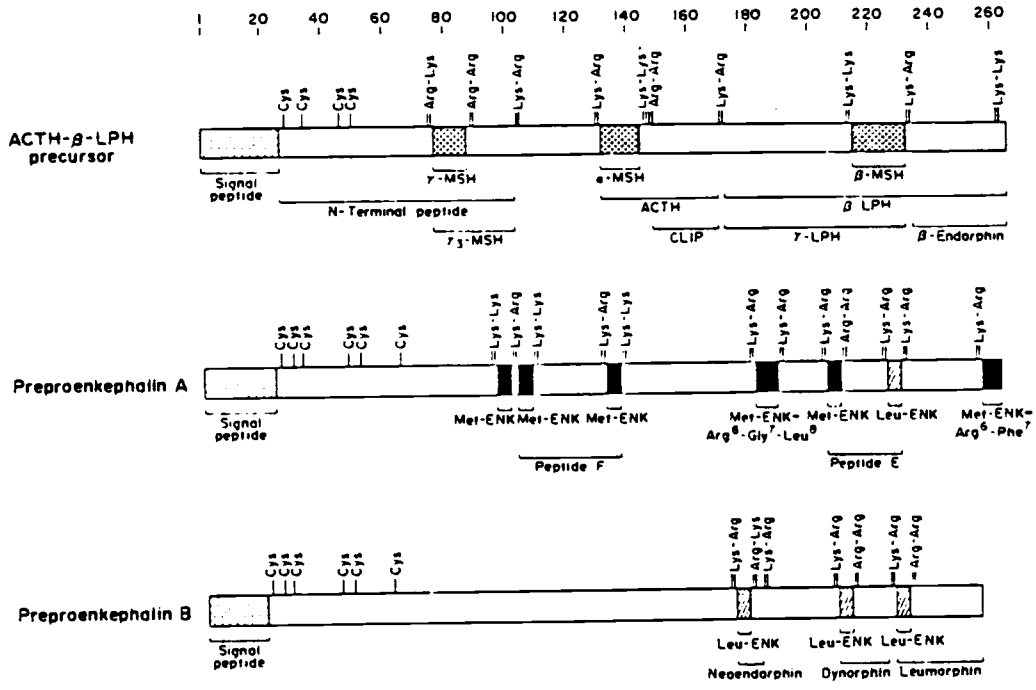


Figure 2.6 Protein Processing

Source: Numa, S. In *The Peptides: Analysis, Synthesis, Biology*; Udenfried, S.; Meienhofer, J., Eds.; Academic: Orlando, 1984; Vol. 6, p 12.

2.4 Dynorphin

Dynorphin A-(1-17) was first identified and isolated from crude porcine pituitary (Cox et al., 1975). When compared to Leu-enkephalin in the guinea pig ileum (GPI), dynorphin is 700 times more potent and 13 times less sensitive to naloxone (Goldstein et al., 1979). It has also been shown to be an agonist in the mouse vas deferens (MVD) (Wuster et al., 1980a,b) and rabbit vas deferens (RbVD) (Oka et al., 1982). Dynorphin A-(1-13) accounts for essentially all the native peptide's biological activity (Goldstein et al., 1981).

Evidence indicates that dynorphin A-(1-13) is a selective kappa agonist in several tissues (Goldstein, 1984). Dynorphin shows agonism in the guinea pig ileum (Goldstein et al., 1979), in the mouse vas deferens (Goldstein et al., 1979; Höllt, 1986) and rabbit vas deferens (Oka et al., 1982; Goldstein, 1983), but dynorphin is inactive in the rat vas deferens (Höllt, 1986). Dynorphin A-(1-13) amide has good kappa receptor selectivity (μ:δ:κ ratios 30:80:1) (Leslie and Goldstein, 1982) (see Figure 2.7).

Receptor binding selectivity refers to the relative binding affinities of a ligand at different receptor sites. This is calculated using the K_i ratios at each nonpreferred site to that at the preferred site, i.e. the highest affinity site (Goldstein, 1984).

Competing ligand	Binding selectivity profile		
	μ	δ	κ
Putative μ ligands			
Naloxone	[9.14]	18	4.0
Normorphine	[8.15]	56	18
Sufentanil	[10.30]	70	220
Morphiceptin	[6.97]	270	86
[DAla^2,MePhe^4,Gly-ol^5]enkephalin (DAGO)[b]	[8.48]	150	170
Putative δ ligands			
[DAla^2,DLeu^5]enkephalin (DADLE)	2.1	[8.82]	6,400
[DSer^2,Leu^5]enkephalin-Thr^6(DSLET)[c]	20	[8.74]	> 1,000
2-5 Cyclo[DPen^{2,5}]enkephalin (DPDPE)[d]	780	[8.38]	> 430
Putative κ ligands			
Ethylketazocine (EKC)	7.5	78	[9.96]
Dynorphin A-(1 - 13) amide	30	78	[10.7]
Bremazocine	3.1	19	[10.1]
U50,488[e]	1,300	12,000	[9.14]

[1]The preferred binding site is denoted by square brackets and the K_i is the antilog of that bracketed number. The affinity for the square bracketed site is x times greater than for the nonpreferred site. (For example: naloxone has the highest affinity for the μ binding site, and its binding affinity is 18 times greater than for the δ site and 4 times greater than the κ site.)

Figure 2.7 Binding Selectivity Profile

Source: Goldstein, A. In *The Peptides: Analysis, Synthesis, Biology*; Udenfried, S.; Meienhofer, J., Eds.; Academic: Orlando, 1984; Vol. 6, p 121.

Comparing the ability of naloxone to antagonize the compounds' effects on GPI demonstrates that dynorphin A-(1-13) is significantly different from morphine. The data suggest that dynorphin A-(1-13) interacts quite distinctly from other opioid peptides in the GPI (Vaught, 1981). Wuster demonstrated that tissues made tolerant to kappa agonists are also tolerant to dynorphin A-(1-13), whereas tissues tolerant to μ or δ agonists are not tolerant to dynorphin A-(1-13) (Wuster et al., 1980a,b).

Goldstein coined the "message" and "address" sequences of dynorphin (Figure 2.8). The N-terminal segment, dynorphin-(1-4) is designated as the "message" sequence (Chavkin and Goldstein, 1981) because it is the shortest fragment with typical naloxone-reversible opioid activity (Schwyzer, 1977). However, the tetrapeptide pocket of the kappa receptor seems to be different from that of the delta receptor, indicated by the differing affinities for naloxone and by the effect of D-Ala^2 on receptor specificity (Chavkin and Goldstein, 1981). In Leu-enkephalin, D-Ala^2 substitution increases potency, but in dynorphin A-(1-13) amide it decreases potency (Chavkin and Goldstein, 1981). The "address" sequence is the remainder of the dynorphin molecule and is responsible for the specificity and the high potency of dynorphin (Chavkin and Goldstein, 1981).

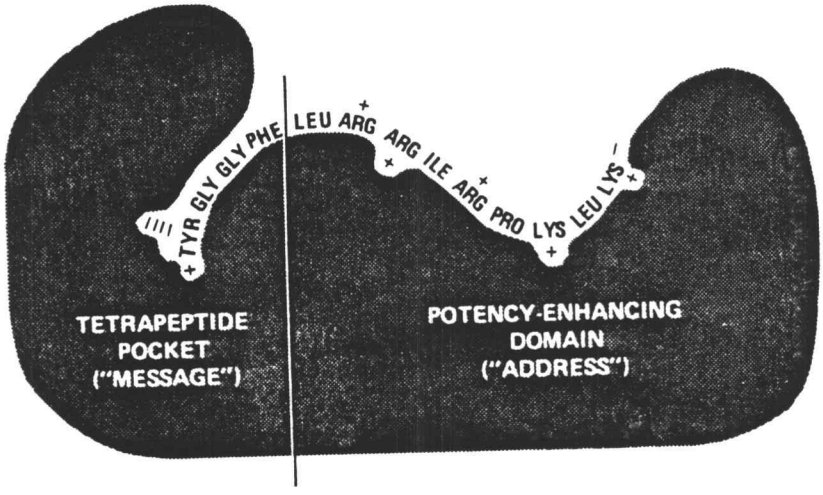


Figure 2.8 Dynorphin and its Receptor

Source: Chavkin, C.; Goldstein, A. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, p 6546.

2.5 Conformation of Dynorphin

The N-terminal enkephalin sequence is the common element required by all opioid receptors. In the enkephalins, the flexibility of the sequence permits a β-bend at Gly^2-Gly^3. This β-bend allows the benzene rings of Tyr^1 and Phe^4 to be in close proximity (Goldstein and James, 1983). The extension of Leu-enkephalin to dynorphin A-(1-13) causes a change in receptor selectivity from δ to κ (Goldstein and James, 1983).

The literature contains contradictory evidence about the conformation of dynorphin. A circular dichroism study of dynorphin's conformation was one of the first reported in the literature. It suggested that dynorphin A-(1-13) showed very little order in an aqueous environment (Maroun and Mattice, 1981). A fluorescence energy study of Trp-substituted dynorphin analogues suggested a predominately extended conformation (Schiller, 1983).

However, laser raman spectroscopy suggested that an aqueous dynorphin A-(1-13) solution has a mixture of extended β-pleated sheet and "random" conformation (Rapaka et al., 1987). A study combining FT-IR, Raman and NMR techniques supported Rapaka's conclusions that dynorphin A-(1-13) assumes a mixture of extended and "random" conformations in aqueous solution (Renugopalakrishnan et al., 1988a,b).

The amphiphilic environment of an aqueous solution in combination with a lipid bilayer of a membrane could impose secondary structure on

dynorphin (Schwyzer, 1986a,b). A study of dynorphin A-(1-13), using the technique of vesicle-mediated hydrophobic photolabeling, revealed that the basic dynorphin, with a net charge of +5, interacts with anionic liposomes. The N-terminal "message" sequence interacts with the hydrophobic membrane layers while the "address" sequence remains in the aqueous phase (Gysin and Schwyzer, 1983).

Schwyzer's group proposed a "membrane-assisted" model for the interaction of dynorphin with its kappa receptor. In this model, Tyr^1 through Pro^{10} of dynorphin A-(1-13) adopts a helical conformation oriented perpendicularly to the membrane surface (Erne et al., 1985) (see Figure 2.9).

Infrared attenuated total reflection (IR-ATR) spectroscopy and capacitance minimization (CM) indicated that dynorphin A-(1-13) assumed a helical structure oriented perpendicular to the surface of the neutral lipid membrane prepared from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (Erne et al., 1985). This suggests that the behavior of the amphiphilic peptide in contact with neutral lipid membranes may be quite different from that in aqueous solution (Erne et al., 1985; Schwyzer, 1986a,b). A modified conformation has been proposed for dynorphin, in the presence of egg lysolecithin vesicles, which contains an α-helix between Phe^4 and Arg^9 (Renugopalakrishnan et al., 1988a,b).

A recent paper using proton NMR and circular dichroism to study the conformation of a model peptide for dynorphin A-(1-17) (Tyr-Gly-Gly-Phe-

Leu-Lys-Lys-Val-Lys-Pro-Lys-Val-Lys-Val-Lys-Ser-Ser) suggests that an amphiphilic β-strand conformation might be adopted by dynorphin A residues 7-15 on cell surfaces and that this β-strand conformation controls the receptor binding selectivity of the enkephalin segment (Vaughn and Taylor, 1989).

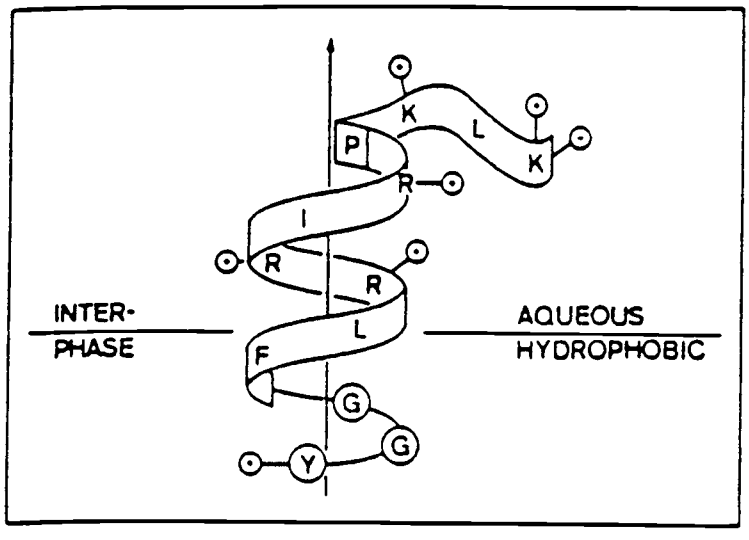


Figure 2.9 Model of Dynorphin A-(1-13) Conformation

Source: Erne, D.; Sargent, D.F.; Schwyzer, R. *Biochemistry* **1985**, *24*, p 4263.

2.6 Metabolism of Dynorphin

Dynorphin metabolism is rapid and complex both *in vivo* and *in vitro* (see Figure 2.10). This metabolism problem complicates the evaluation of opioid activity and receptor binding. The degradation of dynorphin can be decreased by lowering the assay incubation temperatures and using enzyme inhibitors, but can't be eliminated (Leslie and Goldstein, 1982).

Metabolism of dynorphin can occur at several sites. The metabolized fragments may be inactive or shorter active peptides. The shorter active peptides may have a different biological profile from the parent peptide.

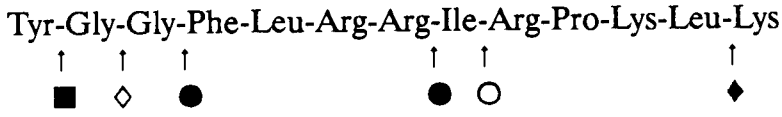
2.6.1 N-terminal Dynorphin Metabolism

Metabolism in the amine terminus inactivates the peptide. Tyr^1 is liberated from dynorphin by aminopeptidase M (EC 3.4.11.2) (Marks et al., 1986). This type of degradation could be inhibited by bestatin but not by captopril (Churchill et al., 1987). The N-terminal dipeptide, Tyr-Gly, can be cleaved from dynorphin by a diaminopeptidase. Both aminopeptidase and diaminopeptidase activities are lower with the longer dynorphin A-(1-13) and dynorphin A-(1-17) as compared with the shorter fragment dynorphin A-(1-8) (Leslie and Goldstein, 1982). The N-terminal tripeptide, Tyr-Gly-Gly, is cleaved from the parent by a metalloendopeptidase (EC 3.4.24.11), commonly known as enkephalinase.

Neither dynorphin A-(1-17) nor dynorphin A-(1-13) is metabolized by angiotensin converting enzyme (ACE), yet dynorphin A-(1-8) is a good substrate for ACE (Benuck et al., 1984).

2.6.2 C-terminal Dynorphin Metabolism

Metabolism in the carboxyl terminus of dynorphin yields biologically active peptide fragments. Lys^{13} is cleaved from dynorphin A-(1-13) by a carboxypeptidase (Leslie and Goldstein, 1982). Endopeptidase cleavage results in shorter peptide fragments that are less selective for the kappa receptor (Goldstein, 1984). Metalloendopeptidase (enkephalinase) cleaves at both the Gly^3-Phe^4 and Arg^7-Ile^8 bonds (Shaw et al., 1982). An endopeptidase present in synaptosomal membranes converts both dynorphin A-(1-17) and dynorphin A-(1-13) to dynorphin A-(1-8) (Benuck et al., 1984). Dynorphin A-(1-8) is more susceptible to enzymatic degradation than dynorphin A-(1-17) or dynorphin A-(1-13) (Corbett et al., 1982).



Exopeptidases

- ■ aminopeptidase
- ◇ diaminopeptidase
- ♦ neutral carboxypeptidase

Endopeptidases

- ● metalloendopeptidase
- ○ synaptic endopeptidase

Figure 2.10 Metabolism of Dynorphin A-(1-13)

Adapted from Marks, N.; Benuck, M.; Berg, M.J. *Natl. Inst. Drug Abuse Res. Monogr.* **1986**, *70*:68.

2.7 Structure-Activity Relationships of Dynorphin-A-(1-13) Analogues

2.7.1 Agonist Activity

There have been relatively few structure-activity relationship studies and to the best of my knowledge there have been no molecular modelling studies on dynorphin. The structural requirements that impart biological activity to the molecule are important pieces of information. This type of information also sheds light on receptor structure and function. With dynorphin, it has been shown that the N-terminal Tyr is essential for opioid activity (Leslie and Goldstein, 1982), whereas the basic Arg^7 and Lys^{11} residues make the greatest contribution toward potency and selectivity (Chavkin and Goldstein, 1981; Goldstein, 1983). Shortening dynorphin A-(1-17) to dynorphin A-(1-11) increased kappa binding selectivity and decreased the binding affinity for delta and mu receptors (Goldstein and James, 1983) (see Appendix).

2.7.1.1 "Message" Sequence Modifications

Amino acid replacement of Tyr^1, Gly^2, Phe^4 and Leu^5 with Ala substantially decreased opioid activity and binding (Turcotte et al., 1984). When Gly^2 was replaced with D-Ala there was a decrease in opioid activity and kappa receptor selectivity (Chavkin and Goldstein, 1981). Replacement of Phe^4 by $Phe(NO_2)$ only slightly decreased opioid activity while the data

suggest that the analogue is still selective for the kappa receptor (Schiller et al., 1982). Phe^4 replacement with Trp also showed decreased opioid activity (Schiller, 1983).

The conformationally constrained [$D\text{-}Cys^2$,Cys^5] analogue is more potent than the linear parent, but receptor selectivity (Shearman, 1985) shifted from kappa to delta (Schiller et al., 1982). Other cyclic analogues containing an amide bond between Orn and Asp showed mu receptor selectivity (Schiller et al., 1988).

2.7.1.2 "Address" Sequence Modification

Stereoisomeric replacement of Arg^6 had little effect on kappa receptor selectivity (Wuster et al., 1980a,b). Replacement of the basic residues with Ala caused a larger decrease in opioid activity than did Ala substitution at Ile^8 or Pro^{10} (Turcotte et al., 1984). Replacement of Ile^8 by either Ala isomer showed a high binding affinity (Turcotte et al., 1984) and greater kappa selectivity (Lemaire et al., 1986) than did the parent compound. Stereoisomeric replacement of Pro^{10} increased kappa receptor selectivity (Lemaire et al., 1986) and in the dynorphin A-(1-11) compound, increased binding affinity (Gairin et al., 1985). Either isomer of Trp in position 8 or 10 resulted in both decreased opioid activity and binding affinity (Lemaire et al., 1986).

A novel synthetic probe, "DAKLI", was prepared from dynorphin A-(1-13). This ligand, [$Arg^{11,13}$]dynorphin A-(1-13)-Gly-$NH(CH_2)_5NH_2$ (Dynorphin A-analogue Kappa LIgand), has a high affinity and selectivity for the kappa receptor. The reactive amino group at the C-terminus allows for a variety of functional groups to be attached. The dissociation constant of [^{3}H]bremazocine/U-50,488 against dynorphin A-(1-13)NH_2 was 4.79 x 10^{-11} while the dissociation constant of [^{125}I]DAKLI/U-50,488 was 8.71 x 10^{-11} (Goldstein, 1988). U-50,488 was used to define specific binding in both cases.

Another interesting analogue of dynorphin is [biocytin^{13}]dynorphin A-(1-13)NH_2. This analogue is biotinylated on the ε-amine of Lys^{13}. This analogue has only a slightly reduced binding affinity for the κ receptor but binds very tightly to it. This type of ligand can be used in the biotin-avidin system as a κ receptor probe (Hochhaus et al., 1988).

2.7.2 Antagonist Activity

At the present only three papers have reported dynorphin analogues with antagonist activity. The disubstituted [Ala^2,Trp^4]dynorphin A-(1-13) analogue exhibited significant antagonist activity (Lemaire et al., 1986). Two of the trisubstituted [D-$Trp^{x,8}$,D-Pro^{10}] analogues showed moderate antagonist activity and three only showed weak antagonism. Most recently, two N,N-diallyl dynorphin analogues showed antagonism (Gairin et al., 1988).

2.8 Peptide Synthesis

Peptides have been shown to play key roles in neurological, pharmacological, and physiological function. Rapid, efficient and reliable synthesis is necessary in order to further study the role of peptides in biological processes (Barany and Merrifield, 1979). The art and science of peptide synthesis was pioneered by Emil Fischer and Theodor Curtius in the early 1900's and since that time has become a discipline of great power and sophistication (see Bodanszky, 1988).

2.8.1 Solid Phase Synthesis

The solid-phase approach to peptide synthesis was conceived in 1959 by Bruce Merrifield in an attempt to circumvent the inherent problems of peptide synthesis in solution (Merrifield, 1962). Solution chemistry requires skill and time, and is labor intensive. The large amount of resources needed are predominately attributable to the unpredictable solubility of the product and the necessity of intermediate purification (Barany et al., 1987). The concept of solid phase peptide synthesis was introduced when the first peptide was prepared by this method in 1963 (Merrifield, 1963).

The solid-phase process involves the attachment of a growing peptide chain to an insoluble polymeric resin. By attaching the peptide to an insoluble resin any by-products or unused reagents can be removed by

filtration and solvent washes. Amino acid residues are added to the growing peptide chain in a cycle that consists of successive coupling and deprotection reactions (see Figure 2.11). This method has been automated in order to increase efficiency and decrease the time necessary to produce a peptide.

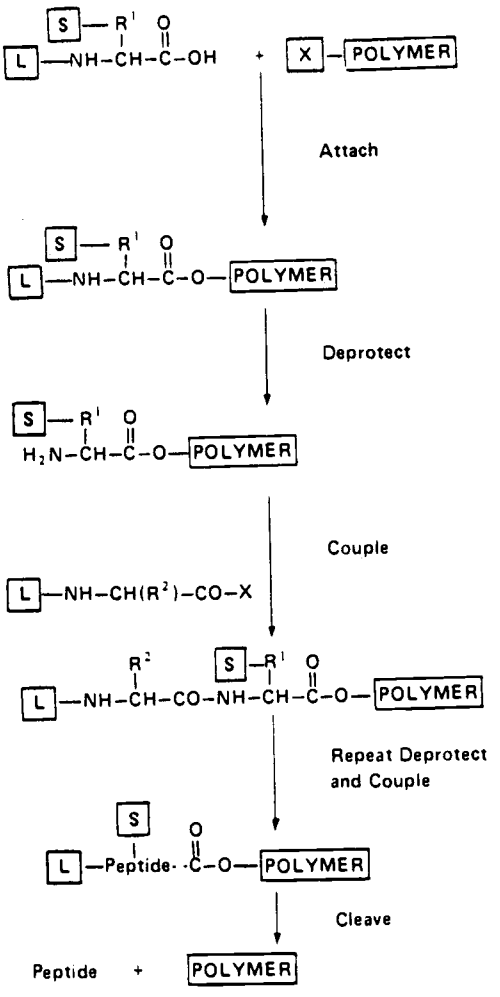


Figure 2.11 Solid Phase Synthesis Scheme

Source: Stewart, J.M.; Young, J.D. *Solid Phase Peptide Synthesis*, 2nd ed.; Pierce Chemical: Rockford, 1984; p 2.

2.8.2 Boc Protection Strategy

A temporary protecting group is obligatory for the N^{α}-amine of all amino acids to be coupled (Barany and Merrifield, 1979). The Boc (t-butyloxycarbonyl) N^{α}-amine protecting groups is used in conjunction with Merrifield's classical solid phase synthesis technique (see Figure 2.12). Boc is an acid-labile protecting group, removed using a 50% trifluoroacetic acid (TFA) solution, which was first introduced in the late 1950's by two independent groups (McKay and Albertson, 1957; Anderson and McGregor, 1957).

The use of the Boc chemical protocol involves the simultaneous use of a differential acid-stable protecting group for any reactive side chains of the amino acid residues being coupled. Side chain protecting groups typically used include a benzyl ester (OBzl) for Asp or Glu; 2-chlorobenzyloxycarbonyl (2-Cl-Z) for Lys; 2,6-dichlorobenzyl (Cl_2Bzl) for Tyr and 4-methylbenzyl (MeBzl) for Cys (Stewart and Young, 1984). These protecting groups require a strong acid for deprotection. The p-toluenesulfonyl, (Tos) side chain protecting group for Arg or His requires an even stronger acid deprotection, HF, for removal (Fauchère and Schwyzer, 1981).

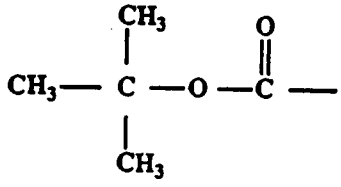


Figure 2.12 Boc Protecting Group

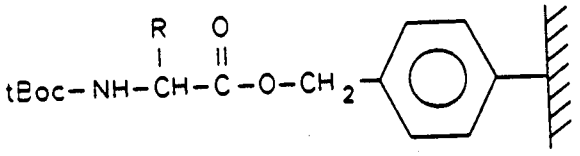
2.8.3 Resins Used in Boc Chemistry

The final component in the Boc chemical protocol is the solid support or resin. This resin is typically a polystyrene-based polymer cross-linked with divinylbenzene. Three different resins are most commonly used in the Boc chemical protocol (see Figure 2.13). The standard Merrifield resin (**1**), which produces a peptide acid upon HF cleavage, is a chloromethylatedphenyl polystyrene support which upon reaction with a protected amino acid produces a benzyl ester linkage.

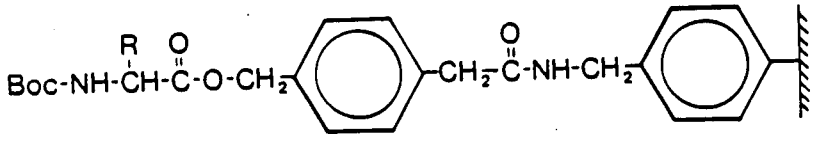
The second type of resin is the PAM, 4-(oxymethyl)-phenylacetamidomethyl) resin (**2**), which also produces a peptide acid upon HF cleavage and is often used for larger peptides because of the increased stability of the peptide-resin linkage.

The third type of solid support, the benzhydrylamine resin, produces peptide amides upon HF cleavage. The most commonly used resin for the synthesis of a peptide amide is the 4-methylbenzhydrylamine (MBHA) resin (**3**). The substitution of the methyl group in the benzhydrylamine resin increases the acid lability of the resin (Barany and Merrifield, 1979). A peptide amide may also be prepared by using a polystyrene resin with a benzyl ester linkage, but the benzyl ester linkage requires cleavage by methanolic ammonia to form a peptide amide while the MBHA resin is acid-labile. The MBHA resin is also advantageous over the benzyl ester resin because of the inability for methyl ester formation.

(1)



(2)



(3)

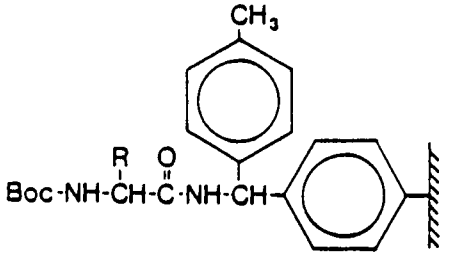


Figure 2.13 Resins Used in Boc Chemistry

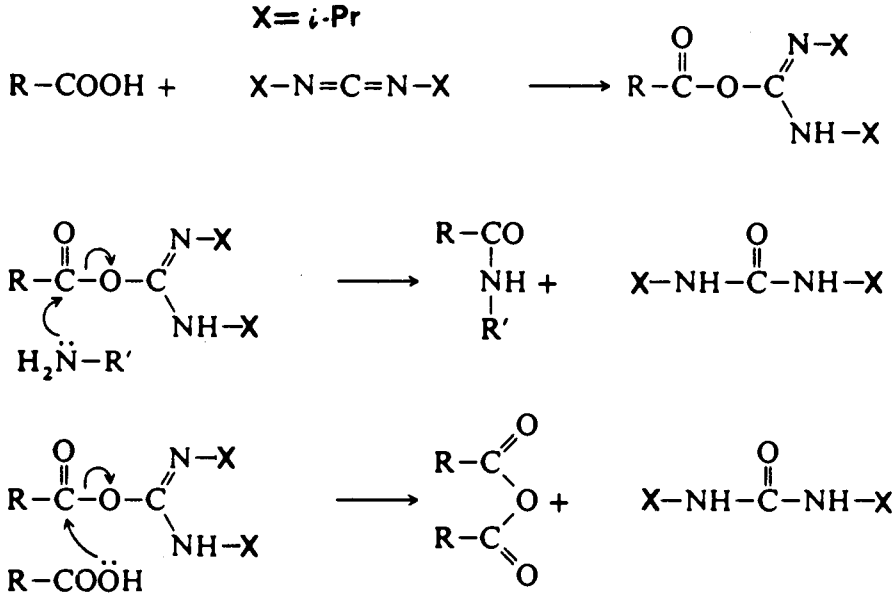
Source: Milligen/Biosearch Model 9500 Operator's Guide.

2.8.4 Coupling Mechanism

One of the most common methods for coupling the incoming protected amino acid residue to the growing peptide chain is to use a carbodiimide (e.g. DIPCDI). The carbodiimide reacts with the carboxylic acid which attacks the carbon-nitrogen double bond in the carbodiimide to form the reactive O-acylisourea (Figure 2.14). The by-product upon subsequent reaction is a urea which is removed. DIPCDI is advantageous over the previously used carboxylic acid activator, dicyclohexylcarbodiimide (DCC), because the urea produced from DCC is an insoluble solid where the byproduct of DIPCDI is a soluble urea.

1-Hydroxybenzotriazole (HOBt) can be used as a coupling additive with carbodiimides which converts the O-acylisourea into a more stable, but equally reactive benzotriazolyl ester (Hudson, 1988) which promotes effective coupling by allowing longer coupling times (Figure 2.14).

DIPCDI Coupling



DIPCDI + HOBt Coupling

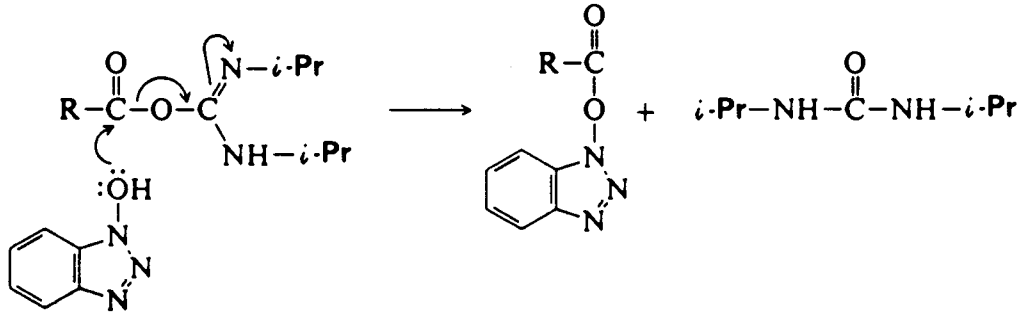


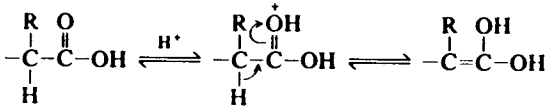
Figure 2.14 Coupling Reaction Mechanism

Adapted from: Bodanszky, M. *Peptide Chemistry*; Springer: Berlin; 1988, p 159.

2.8.5 Racemization

Amino acids residues are added to the solid support stepwise in the C-terminal --> N-terminal direction to prevent racemization. The base-catalyzed racemization results from enolization with the loss of stereochemistry around the chiral center of the amino acid producing a racemic mixture or oxazolone formation (Kemp, 1979). The use of a urethane-based protecting group, such as Boc, reduces the probability for racemization by the oxazolone mechanism (see Figure 2.15). HOBt was also used during coupling to prevent racemization. It acts as a second nuclephile which reduces the effective concentration of the O-acylisourea, and being a weak acid, its donatable proton prevents proton abstraction from the amino acid's chiral center.

Enolization



Oxazolone Formation

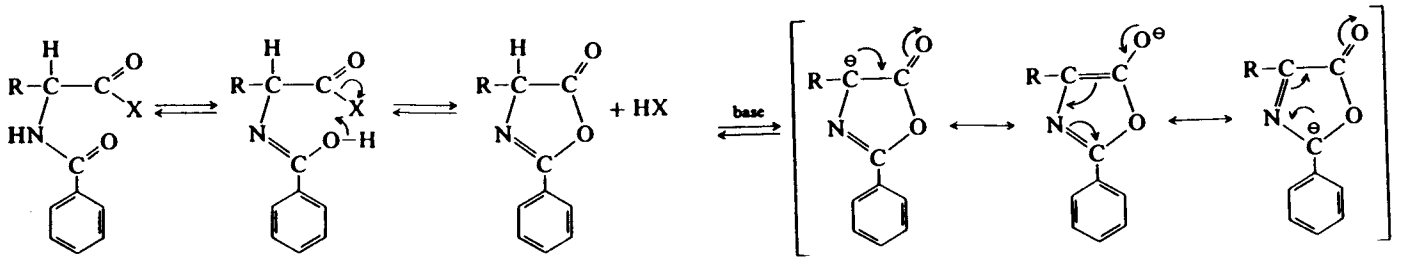


Figure 2.15 Racemization Mechanisms

Source: Bodanszky, M. *Peptide Chemistry*; Springer: Berlin, 1988; p 116.

CHAPTER 3

OBJECTIVES AND RATIONALE

3.1 Objectives

The objectives of Part One of this thesis research were to synthesize and purify a series of 2-substituted dynorphin A-(1-13) amide analogues (see Figure 3.2). It was hypothesized that these compounds would give a better understanding of the interaction of dynorphin with the kappa opioid receptor at the molecular level.

Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-LysNH_2

Figure 3.1 Structure of Dynorphin A-(1-13)NH_2

The peptides were assembled by solid phase peptide synthesis using the Boc chemical protocol on a 4-methylbenzhydrylamine (MBHA) resin. The peptides were cleaved from the polystyrene resin using HF and purified using gel filtration and reverse phase preparative HPLC. The compounds were assayed for purity by amino acid analysis, fast atom bombardment mass

spectrometry (FAB-MS) and analytical HPLC. The peptides were tested for pharmacological activity in smooth muscle preparations by Dr. Gary DeLander and in radioligand binding assays by Dr. Thomas Murray.

Tyr-X-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-LysNH_2

X	=	Gly	(Dynorphin A-(1-13)NH_2)
		Asn	
		Leu	
		Lys	
		Met	
		Ser	
		D-Asn	
		D-Leu	
		D-Lys	
		D-Met	
		D-Ser	

Figure 3.2 Compounds Synthesized

3.2 Rationale

3.2.1 Choice of Dynorphin A

The analgesia of dynorphin is a result of the peptide's interaction with kappa opioid receptors in the spinal cord (Goldstein, 1983). This is a complex physiological and pharmacological process that encompasses more than

analgesia. Thus, it is important to define further the functions of the kappa opioid receptors.

The opioid peptide dynorphin A-(1-13)NH_2 was chosen because the first thirteen residues account for most of the biological activity of the endogenous dynorphin A-(1-17). The dynorphin amide was used because it showed improved metabolic stability over the endogenous acid, dynorphin A-(1-17), while still retaining kappa receptor selectivity (μ:δ:κ = 30:80:1) (Goldstein, 1984).

The preparation of dynorphin analogues is important for several reasons. Given the structure of the peptide, a large number of analogues can be synthesized by modification of the individual amino acid residues making up the peptide. Extensive evaluation of structure-activity relationships can be conducted in an attempt to develop analogues with a desired biological activity. The structure-activity relationships for different amino acid residues provide information on what structural features in the peptide are important for a given biological activity and also can lend insight into the structure of the receptor and its interaction with peptides as compared to the opiates.

3.2.2 Choice of 2 Position for Substitution

The 2 position was chosen for substitution for two reasons. First, the Gly^2 residue is part of the N-terminal enkephalin "message" sequence that is required for opioid activity (Goldstein, 1984). The goal of this research was

to determine how an alteration in the Gly^2 position would change the activity of the peptide. This modification is based on the hypothesis that alterations in the "message" portion of dynorphin could change opioid activity from agonist to antagonist as well as affect potency, while changes in the "address" sequence would only alter analogue potency and/or receptor selectivity (Goldstein, 1984). To test this hypothesis the N-terminal portion of dynorphin A-(1-13)NH_2 was modified at the 2 position by replacing glycine with various amino acid residues. The [Ala^2,Trp^4]dynorphin A-(1-13) analogue was found to show antagonist activity (Lemaire et al., 1986). Additionally, peptides containing D-Trp^2 have also shown antagonism (Lemaire and Turcotte, 1986). Therefore modifications at the 2-position of dynorphin A were examined in more detail.

Second, this residue could be important for determining the conformation of the "message" sequence of dynorphin. This residue has been used as a site for a conformationally constrained [D-Cys^2, Cys^5]dynorphin A-(1-13) analogue. Unfortunately, this cyclic peptide did not show kappa receptor selectivity (Shearman, 1985). This may indicate that an unfavorable steric or electronic interaction is produced by the constraint, but more likely the lack of kappa receptor selectivity is due to the wrong conformation imposed by the constraint which decreases affinity for the kappa receptor. Substitutions at the Gly^2 position might alter the relative orientation of the important Tyr^1 and Phe^4 residues. The conformation of the N-terminal

tetrapeptide, Tyr-Gly-Gly-Phe, of Leu-enkephalin is folded whereas the same N-terminal segment of dynorphin A-(1-13) is almost completely extended (Goldstein and James, 1983). This suggests that a change in the conformation accompanying the extension of Leu-enkephalin effects opiate receptor selectivity (Schiller, 1983).

3.2.3 Choice of Amino Acids for Substitution

The range of amino acids chosen for this study were picked based on their ability to affect the conformation of the peptide (Chou and Fasman, 1974), the charge on the position, and hydrophobicity of the analogue (Kyte and Doolittle, 1982).

3.2.4 Choice of L-Amino Acids

The amino acids were chosen to examine Schwyzer's proposal that the N-terminus of dynorphin A was in a helical conformation interacting with kappa receptors (Gysin and Schwyzer, 1983). The nonpolar amino acids, Leu and Met, were chosen to replace Gly^2 because of their tendency to form an α-helix (Creighton, 1983) (see Figure 3.3). For comparison, the polar amino acids, Ser and Asn, were chosen because of their low tendency for α-helix formation. These residues, like Gly, prefer to form a β-turn (Creighton, 1983) (see Figure 3.3). Lys was chosen primarily to investigate whether a basic

residue would be tolerated in the "message" sequence. It also has the ability to form an α-helix (Creighton, 1983) (see Figure 3.3).

Amino acid	*α helix*	*β sheet*	*β-turn*
Ala	1.29	0.90	0.78
Cys	1.11	0.74	0.80
Leu	1.30	1.02	0.59
Met	1.47	0.97	0.39
Glu	1.44	0.75	1.00
Gln	1.27	0.80	0.97
His	1.22	1.08	0.69
Lys	1.23	0.77	0.96
Val	0.91	1.49	0.47
Ile	0.97	1.45	0.51
Phe	1.07	1.32	0.58
Tyr	0.72	1.25	1.05
Trp	0.99	1.14	0.75
Thr	0.82	1.21	1.03
Gly	0.56	0.92	1.64
Ser	0.82	0.95	1.33
Asp	1.04	0.72	1.41
Asn	0.90	0.76	1.28
Pro	0.52	0.64	1.91
Arg	0.96	0.99	0.88

Figure 3.3 Relative Frequencies of Occurrence of Amino Acid Residues in the Secondary Structure of Proteins

Source: Creighton, T.E. *Proteins: Structures and Molecular Principles*. W.H. Freeman: New York, 1983; p 235.

3.2.5 Choice of D-Amino Acids

The corresponding D-amino acids were also incorporated into dynorphin A-(1-13)NH_2. Changing the stereochemistry of the amino acid substituent at the Gly^2 position might significantly alter the orientation between Tyr^1 and Phe^4 residues. Such a modification could potentially change the biological profile from agonist to antagonist by altering the way the peptide fits into the receptor. The incorporation of D-amino acids increases peptide stability by inhibition of metabolism (Lemaire et al., 1986b).

CHAPTER 4

EXPERIMENTAL

4.1 Instrumentation and Reagents

The peptides were synthesized on a Biosearch 9500 automated peptide synthesizer (Novato, CA), and cleaved using a Multiple Peptide Systems HF Apparatus Model 2010C (San Diego, CA). The peptides were analyzed and purified using a Beckman Model 431A high performance liquid chromatography (HPLC) system. This system consisted of a Model 421A controller, two Model 110B pumps, Model 201A injector, Model 163 UV detector, Waters Model 740 data module and solvent system. The HPLC analytical column used with the Beckman system was a Dupont Zorbax Protein Plus [300 Å, 6μm, 4.6mm × 25cm] with a Protein Plus guard cartridge. The preparative column used with the system was also a Dupont Zorbax Protein Plus [300 Å, 10μm, 21mm × 25cm] with a Dynamax [21mm × 5cm, C_4 12μm] guard cartridge. The fraction collector used during preparative purification was an ISCO, Model Retriever IV. The lyophilizer used for purification was a Thermovac Model FD-6.

The reagents used in peptide synthesis were: MBHA resin, HOBt, DIPEA and all amino acids except BocTyr and BocD-Asn (Milligen/Biosearch, Novato, CA); BocTyr and BocD-Asn (Sigma); trifluoroacetic acid (TFA) (Kali-Chemie, Greenwich, CT); anisole, ethanedithiol, DIPCDI, and 1-acetylimidazole (Aldrich); and the solvents DCM (methylene chloride), DMF (dimethylformamide), MeOH (Merck, Omnisolv). The reagents and supplies used in purification were: Sephadex G-10 (Piscataway, NJ); acetic acid (Baker); acetonitrile (AcCN) (Burdick & Jackson); TFA (Pierce Sequanal grade in amber ampules); HPLC-grade water (Milli-Q system, College of Pharmacy); Gelman FP Vericel HPLC membrane filter, 0.45 μm pore size, 47 mm diameter (VWR); and Syrfil disposable HPLC filter, 0.45 μm pore size, 25 mm diameter (VWR).

The peptides were analyzed for purity using HPLC, FAB-MS and amino acid analysis. The FAB-MS was done by the Department of Agricultural Chemistry of Oregon State University, Corvallis, OR. The amino acid analysis was done by the Protein Structure Laboratory of the University of California, Davis, CA.

4.2 General Procedures

4.2.1 Solid Phase Synthesis

The peptides were synthesized using the Boc chemical protocol (Milligen/Biosearch 9500 operators manual) on an MBHA (4-methylbenzhydrylamine) resin (substitution of 0.36 mmoles per gram of resin). This polystyrene-based resin is crosslinked to about 1% with divinylbenzene and is normally in the range of 200 - 400 mesh. In preparation for synthesis, the resin was swollen in about 20 mL of DCM for 10 minutes. After the resin was swollen, the synthesis began by washing the resin 2 × with 20 mL of DCM, neutralizing the resin 3 × with 20 mL of a base wash containing 10% DIPEA in DCM. The resin was washed 5 × with 20 mL of DCM and 3 × with 20 mL of DCM/DMF (1:1).

The first amino acid, BocLys(2-Cl-Z), was double coupled to the resin using a 0.4 M amino acid solution in DMF, 6.67-fold excess, with 0.4 M DIPCDI in DCM in equal amounts, for two hours. The resin was washed 7 × with 20 mL with DCM and 5 × with 20 mL of DMF. Following the coupling reaction, the resin was treated with 0.3 M acetylimidazole in DMF for 30 minutes to block any unreacted amin(1.5e. The resin was washed with 2 × with 20 mL of DMF and 4 × with 20 mL of DCM.

The repetitive cycle for synthesis of the remaining residues began with a deblock treatment. The resin was reacted with the deblock solution, 45%

TFA, 2.5% anisole and 52.5% DCM, to remove the temporary Boc protecting group. The resin was deblocked for one minute as a pretreatment then reacted with the TFA solution for twenty minutes. Following deblock, the resin was washed 2 × with 20 mL of DCM and 3 × with 20 mL of DCM/DMF (1:1) and neutralized as described above. The next amino acid residue was coupled with DIPCDI and treated with acetylimidazole as previously described and the cycle continued until the dynorphin analogue was assembled. HOBt (1.5 equivalents) was added to the BocGly in all analogues.

The side chain protecting groups used were tBu for the hydroxyl of Ser; Tos for the guanidinium of Arg; and 2-Cl-Z for the e-amine of Lys. The phenol of the N-terminal Tyr was not protected.

When the synthesis was completed the resin was washed with DMF, DCM/DMF (1:1), DCM and shrunk with MeOH. The resin was dried *in vacuo* in preparation for cleavage.

4.2.2 Cleavage

The peptide was transferred to a reaction vessel of the HF apparatus and 1 mL of anisole was added as a carbonium ion scavenger. After purging the system with nitrogen, the reaction vessels were cooled to -70°C for 5-10 minutes. Upon cooling, the HF was condensed in the reaction vessels to reach a volume of at least 10 mL; the condensation took about 20 min. After the condensation of HF was complete, the dry ice bath was replaced with an

ice water bath and the cleavage reaction continued with stirring for 50 - 60 minutes at 0°C. The HF was removed under a stream of nitrogen; base traps containing a 20% KOH solution were used to neutralize the volatilized HF. The reaction vessel was aspirated for an hour to remove all remaining HF.

The resin samples were extracted with 3 × 20 mL ether, and were filtered through a fritted funnel using an aspirator in the hood; the samples were dried in a vacuum desiccator to remove the ether. The peptide was extracted from the resin with 3 × 10-20 mL of 10% HOAc and the solution filtered through a fritted funnel. The acetic acid filtrate was lyophilized to recover the peptide.

Multiple peptides were cleaved together, except for the first dynorphin A-(1-13)NH_2 synthesis. The following peptides were cleaved together: [Leu^2]- and [Met^2]dynorphin A-(1-13)NH_2; [Ser^2]-, [Asn^2]- and [Lys^2]dynorphin A-(1-13)NH_2; dynorphin A-(1-13)NH_2, [D-Leu^2]- and [D-Ser^2]dynorphin A-(1-13)NH_2; [D-Met^2]-, [D-Lys^2]-, and [D-Asn^2]dynorphin A-(1-13)NH_2.

4.2.3 Purification

Purification of the crude peptide used two types of chromatography: (1) gel filtration chromatography and (2) reverse phase high performance liquid chromatography.

4.2.3.1 Gel Filtration Chromatography

The crude peptide was desalted and resin debris removed using a Sephadex G-10 gel filtration column (2.6 × 16.3 cm), eluted with degassed 50% HOAc; the elution of the peptide was monitored at 280 nm. The void volume of the Sephadex column was determined by the elution of dextran blue (38 mL). The peptide was lyophilized in preparation for further purification.

4.2.3.2 High Performance Liquid Chromatography (HPLC)

The crude peptides were first analyzed by analytical HPLC. A 1 mg/mL sample of the lyophilized crude peptide was dissolved in HPLC H_2O, filtered to remove any particulates and a 20 μL aliquot injected onto the analytical column. The peptide was eluted using a mobile phase gradient of 100% A (A = 0.1% (w/v) TFA in H_2O) to 70% B (B = 0.1% (w/v) TFA in AcCN) over 35 min at a flow rate of 2 mL/min.

The crude peptide (not more than 100 mg) was dissolved in 1.0 mL of H_2O, either filtered or centrifuged to remove particulates and loaded onto the preparative column. Dynorphin A-(1-13)NH_2 and analogues [Leu^2]-, [Ser^2]-, [Asn^2]- and [Lys^2]dynorphin A-(1-13)NH_2 were eluted from the column using a mobile phase gradient of 100% A to 70% B over 70 minutes at a flow rate of 10 mL/min, with the eluent collected by a fraction collector. The

remaining analogues were eluted from the column using a mobile phase gradient of 100% A to 70% B over 140 minutes at a flow rate of 10 mL/min.

Each purified fraction was examined for purity by injecting 20 μL sample onto the analytical column. The fractions were analyzed using an isocratic system, generally at a flow rate of 2 mL/min. The isocratic system was determined using the retention time of the main peak from the initial analytical gradient. Generally the isocratic condition (% B) = [(Retention time (min) * 2 % / min) - 12 %]. All peak fractions showing a single peak with a concentration of >95% at a consistent retention time were considered pure. These fractions were lyophilized and the resulting peptide was characterized by FAB-MS and amino acid analysis. A sample of the pure peptide was submitted for pharmacological testing.

4.3 Dynorphin A-(1-13) amide

The synthesis used 1.00 grams of MBHA resin; ninhydrin tests after each coupling were negative and yielded a weight gain of 613.2 mg (72.5%). The recovery after HF cleavage was 423.7 mg (68.3%). Purification by the G-10 column gave 338.4 mg (47.9%) and subsequent HPLC purification of 104.2 mg of the peptide yielded 95.8 mg (47.3%).

4.4 L-Analogues of 2-Substituted Dynorphin A-(1-13) amide

The L-analogues were synthesized similiarly to dynorphin A-(1-13)NH_2. Each synthesis used 0.5 g of MBHA resin and the results for these analogues are summarized in Tables 4.1 through 4.3. The [Asn]-analogue required the addition of HOBt (1.5 equivalents) to the BocAsn and the residue was double coupled to the peptide. The deblock solution for the [Met]-analogue was modified to contain 45% TFA, 2.5% anisole, 2% ethanedithiol and 50.5% DCM.

Table 4.1 L-Analogue Yields

	Synthesis		Cleavage		Purification				
					G-10		HPLC		
Analogue	Weight Gain(mg)	% Expected	mg Recovery	% Yield	mg Recovery	% Yield	mg Purified	Recovery	% Yield
Gly^2	613.2*	72.5	423.7	68.3	338.4	47.9	104.2	95.8	37.8
Asn^2	242.5	56.0	225.7	70.5	184.5	50.8	179.1	147.0	35.9
Leu^2	310.4	71.7	186.1	58.1	277.9	76.5	99.2	59.8	39.7
Lys^2	406.1	87.3	294.9	90.5	238.6	63.4	225.8	120.9	28.7
Met^2	323.9	74.4	184.9	57.3	164.1	44.8	128.2	65.6	19.8
Ser^2	377.6	86.2	227.9	72.3	182.6	50.9	167.7	92.1	24.1

* 1 gm synthesis

Table 4.2 HPLC[1] and FAB-MS Data for L-Analogues

Analogue	Gradient Rv (mL)[2]	Isocratic Rv (mL)	%B	FAB-MS M+1
Gly[2]	32.5	9.5	22.5	1603
Asn[2]	33.6	8.9	22.0	1660
Leu[2]	34.7	10.1	26.0	1659
Lys[2]	36.8	9.5	19.0	1674
Met[2]	35.9	13.5	25.0	1677
Ser[2]	36.4	10.8	22.0	1633

[1] Zorbax Protein Plus Column, 0.46 × 25 cm;
Solvent A = H_20 + 0.1% TFA
Solvent B = AcCN + 0.1% TFA.

[2] 0 to 70% B over 35 min at 2 mL/min.

Table 4.3 Amino Acid Analysis of L-Analogues

	Tyr(1)	Gly(1)	Phe(1)	Leu(2)	Arg(3)	Ile(1)	Pro(1)	Lys(2)	Asx	Ser	Met	%*	%+
Gly	0.90	2.05(2)	0.88	2.03	3.18	0.94	0.96	2.06				66.9	95.4
Asn	0.95	1.00	0.94	2.01	3.17	0.94	1.01	1.98	1.02			45.0	63.5
Leu	0.95	1.01	0.95	2.97(3)	3.18	0.94	0.98	2.03				61.2	86.5
Lys	0.95	1.01	0.93	2.01	3.18	0.94	0.96	3.02(3)				47.2	69.7
Met	0.94	1.01	0.93	2.15	3.23	1.00	0.95	2.10		0.69		65.6	92.5
Ser	0.98	1.01	0.95	1.99	2.22	0.94	0.93	2.07			0.91	54.8	77.8

Quantitation

%* = % peptide
%+ = % (peptide + salt)

4.5 D-Analogues of Dynorphin A-(1-13) amide

The precursor synthesis used 1.00 grams of MBHA resin to synthesize dynorphin A-(3-13)NH_2. At the completion of the synthesis, the resin was washed 5 x with base wash and emptied under nitrogen for 10 minutes. Then the resin was divided in half and the synthesis continued with solvent washes. The resin fractions were used to synthesize [D-Leu^2]- and [D-Ser^2]dynorphin A-(1-13) amide (see Table 4.4 for data on yields). Changes for specific peptides were the use of the tBu protecting group for the side chain of D-Ser.

The second precursor synthesis used 1.50 grams of MBHA resin to synthesize dynorphin A-(3-13)NH_2 in the manner described above. At the completion of the synthesis the resin was washed 5 × with base wash and emptied under nitrogen for 10 minutes, as described above; then the resin was divided in thirds and the synthesis continued with solvent washes. This resin was used to synthesize [D-Asn^2]-, [D-Lys^2]- and [D-Met^2]dynorphin A-(1-13) amide (see Table 4.4 for data on yields). Changes for specific peptides include using HOBt in coupling of BocD-Asn and double coupling this residue; using 2-Cl-Z as the ε-amine side chain protecting group for BocD-Lys; and modifying the deblock solution in the [D-Met^2] dynorphin A-(1-13)NH_2 synthesis to contain 45% TFA, 2.5% anisole, 2% ethanedithiol and 50.5% DCM. The [D-Met^2]-, [D-Lys^2]- and [D-Asn^2]analogues were also filtered through a 0.45 μ Vericel membrane before addition to the Sephadex

G_{10} column. The results for these analogues are summarized in Tables 4.4 through 4.6.

Table 4.4 D-Analogue Yields

	Synthesis		Cleavage		Purification				
					G-10		HPLC		
Analogue	Weight Gain(mg)	% Expected	mg Recovery	% Yield	mg Recovery	% Yield	mg Purified	Recovery	% Yield
Gly^2	613.2*	72.5	423.7	68.3	338.4	47.9	104.2	95.8	37.8
D-Leu^2			307.5	96.1	273.7	75.3	77.2	40.8	34.3
D-Ser^2			266.5	84.5	192.3	53.6	71.9	15.3	9.8
D-Asn^2			155.7	48.6	141.0	38.8	146.7	44.9	10.2
D-Lys^2			360.4	110.6	312.7	83.1	68.8	12.3	12.6
D-Met^2			298.5	92.4	259.1	70.7	154.6	87.8	34.7

* 1 gm synthesis

Note: Yields are approximate due to method of synthesis.

Table 4.5 HPLC[1] and FAB-MS Data for D-Analogues

Analogue	Gradient Rv (mL)[2]	Isocratic Rv (mL)	%B	FAB-MS M+1
D-Gly[2]	32.5	9.5	22.5	1603
D-Asn[2]	33.6	13.3	25.0	1660
D-Leu[2]	34.7	12.3	26.0	1659
D-Lys[2]	36.8	6.9	26.0	1674
D-Met[2]	35.9	10.0	25.0	1677
D-Ser[2]	36.4	11.5	23.0	1633

[1] Zorbax Protein Plus Column, 0.46 × 25 cm;
Solvent A = H_2O + 0.1% TFA
Solvent B = AcCN + 0.1% TFA.

[2] 0 to 70% B over 35 min at 2 mL/min.

Table 4.6 Amino Acid Analysis of D-Analogues

	Tyr(1)	Gly(1)	Phe(1)	Leu(2)	Arg(3)	Ile(1)	Pro(1)	Lys(2)	Asx	Ser	Met	%*	%+
Gly[1]	0.90	2.05(2)	0.88	2.03	3.18	0.94	0.96	2.06				66.9	95.4
D-Asn	0.97	0.99	0.91	2.02	3.10	0.98	0.96	2.04	1.03			75.9	107.1
D-Leu	0.96	1.08	0.99	3.03(3)	3.10	0.97	0.86	2.01				45.3	64.0
D-Lys	0.99	1.02	0.99	2.01	3.07	0.96	0.98	2.99(3)				87.3	128.9
D-Met	1.03	1.01	1.03	2.07	3.28	0.97	1.00	2.14			0.48	63.6	89.5
D-Ser	0.99	0.98	1.00	2.01	3.14	0.97	0.97	2.05		0.88		60.4	85.6

Quantitation

%* = % peptide
%+ = % (peptide + salt)

[1] From Table 4.3.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Analogue Synthesis

5.1.1 Protection Strategy

The dynorphin analogues were synthesized with protection of the N-terminal amine, the ε-amine of Lys, the guanidino of Arg, and the hydroxyl of Ser or D-Ser in the analogues containing these amino acids. Most of the amino acids in the sequence were aliphatic or had minimal side reactions associated with them and were left unprotected. The protecting groups used in this scheme used two levels of acid lability.

The Boc group was used as the N^{α}-amine temporary protecting group and was the most acid-labile. The semi-permanent Bzl (**1**) (see Figure 5.1) protecting group was used to protect the primary alcohol of Ser. The reactive N^{ε}-amine of Lys could have been protected using either the Z or the 2-Cl-Z protecting group. The 2-Cl-Z (**2**) (see Figure 5.1) protecting group was chosen because it was more acid stable than Z and thus would not be partially lost during repeated TFA treatments. The Tos (**3**) (see Figure 5.1) protecting group was used to protect the guanidino side chain of Arg. The phenolic hydroxyl of Tyr was not protected because it was the last amino acid added

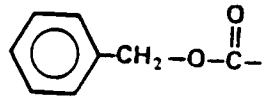
to the growing peptide and the potential for side reactions was considered to be small.

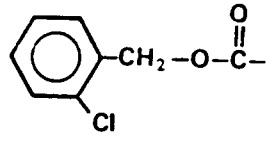
The protecting groups used for the reactive side chains of residues in the synthesis of dynorphin A-(1-13)NH_2 analogues have differing acid labilities. Although the OBzl and 2-Cl-Z groups could have been removed with HBr in HOAc for deprotection, Tos required HF for removal. Thus, HF will also remove the other two more acid labile protecting groups.

5.1.2 Synthesis

The peptide amides were prepared using the MBHA resin. The standard operation of the Biosearch 9500 routinely attaches the C-terminal amino acid residue to the MBHA resin and acylated the resin to block any unreacted amines in preparation for the remainder of the synthesis (see Figure 5.3).

The MBHA resin serves as the C-terminal carboxylic acid protecting group. The -(HN-CH)- linkage of the C-terminal Lys to the MBHA resin is a very acid stable bond and requires HF for liberation.

(1) 

(2) 

(3) 

Figure 5.1 Structure of Side Chain Protecting Groups

BocTyr-X-Gly-Phe-Leu-Arg(Tos)-Arg(Tos)-Ile-Arg(Tos)-Pro-Lys(2-Cl-Z)-Leu-Lys(2-Cl-Z)-NHResin

Figure 5.2 Protected Dynorphin Prototype

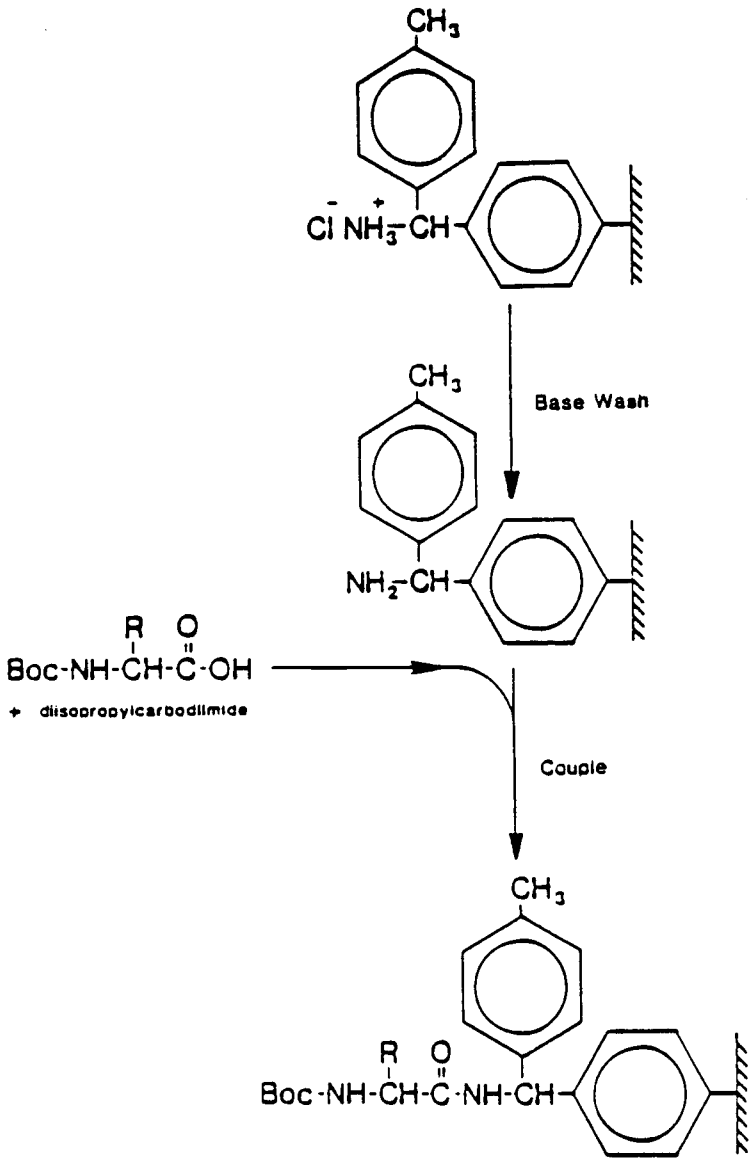


Figure 5.3 Coupling the C-terminal Residue to the MBHA Resin

Source: Milligen/Biosearch Model 9500 Operator's Guide

The synthetic cycle for coupling the amino acid residue to the growing chain consists of four steps: (1) Deblock; (2) Base Wash; (3) Coupling; and (4) Capping (Figure 5.4).

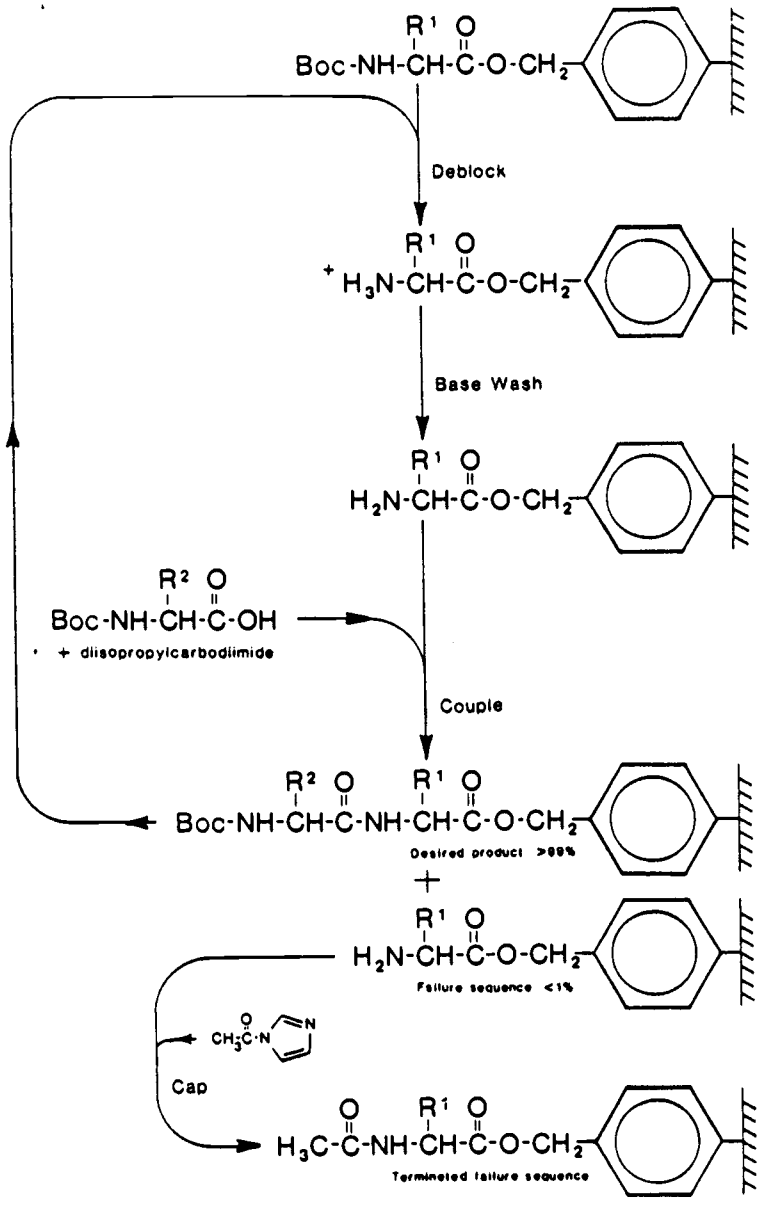


Figure 5.4 Synthesis Cycle Shown on Merrifield Resin

Source: Milligen/Biosearch Model 9500 Operator's Guide

The temporary Boc N^{α}-amine protecting group was removed from the amino acid residue by a solution of TFA. The deblocking cocktail contained anisole to scavenge t-butyl carbonium cations produced during the acid cleavage. Ethanedithiol was used as an additional scavenger for peptides containing a Met residue to protect against alkylation side reactions.

The Base Wash step neutralized the TFA used for deprotection with the secondary amine, diisopropylethlyamine (DIPEA). This neutralization step prevents any potential chain termination caused by trifluoroacetylation during the TFA deblock. The base wash also ensures that the N^{α}-amino groups is deprotonated for the coupling step. The use of a sterically hindered base decreases the potential for racemization.

The incoming protected amino acid residue was coupled to the growing peptide chain using diisopropylcarbodiimide (DIPCDI) or DIPCDI + HOBt. The coupling is performed in a DMF/DCM 1:1 solvent mixture; DCM insures adequate solvation of the polystyrene resin and DMF is necessary in coupling polar sequences. Any base, remaining from the DIPEA wash, present during the coupling reaction can promote base-catalyzed racemization. This racemization potential can be decreased by copious solvent washing prior to coupling to remove any excess base present.

Conversely, any acid present during coupling can promote double insertions caused by the Boc group being removed prematurely. This can be prevented by eliminating the presence of acid during coupling.

Boc is stable to the weakly acidic HOBt present during couplings of BocGly or BocAsn. HOBt was added to BocGly to prevent dimerization during coupling. Gly residues are prone to an amino acid insertion side reaction due to their sterically unhindered side chain (Barany and Merrifield, 1979). HOBt was added to BocAsn and BocD-Asn to compensate for slow coupling kinetics and to suppress side chain dehydration during coupling (see Figure 2.14).

Lys^{13}, the first amino acid added to the resin, was double coupled to insure complete attachment to the resin. If this coupling reaction were incomplete, the unreacted sites would cause shortened peptide sequences. The resin was also capped after this double coupling to insure that any potential reactive site remaining was acylated.

Monitoring coupling reactions is important in peptide synthesis. If the coupling step has not gone to completion the unreacted amine components can lead to deletion sequences. The unreacted amine can be identified using the Kaiser (ninhydrin) test. Any free amine groups on the resin react with ninhydrin to form Ruhmann's purple (see Figure 5.5). The degree of coupling can be determined by the color of the solution; a dark blue (purple) color represents only 76.0% reacted, whereas a yellow color represents > 99.4% reacted (Stewart and Young, 1984).

The ninhydrin test is advantageous over other monitoring methods such as fluorescamine or TNBSA (2,4,6-trinitrobenzenesulfonic acid) because it can

be used to monitor proline. Since Pro is in this sequence, ninhydrin was the method chosen for reaction monitoring in these syntheses.

Ninhydrin tests were done at each residue during the synthesis of dynorphin A-(1-13)NH_2 and all were found to be negative. The ninhydrin test performed at Leu^2 was negative and the test at Tyr^1 of [Leu^2]-dynorphin A-(1-13)NH_2 was slightly positive. The ninhydrin tests performed on [Asn^2]-, [Lys^2]-[Met^2]-, [D-Leu^2]-, and [Ser^2]dynorphin A-(1-13)NH_2 at both the X^2 and Tyr^1 positions were negative. The ninhydrin tests performed on [D-Ser^2]-, [D-Lys^2]-, and [D-Met^2]dynorphin A-(1-13)NH_2 at both X^2 and Tyr^1 positions were a slightly positive green color. Ninhydrin testing was not done on the [D-Asn^2]-analogue. The results from the ninhydrin test suggest that the coupling of the D-isomers was slightly less efficient that their corresponding L-isomers.

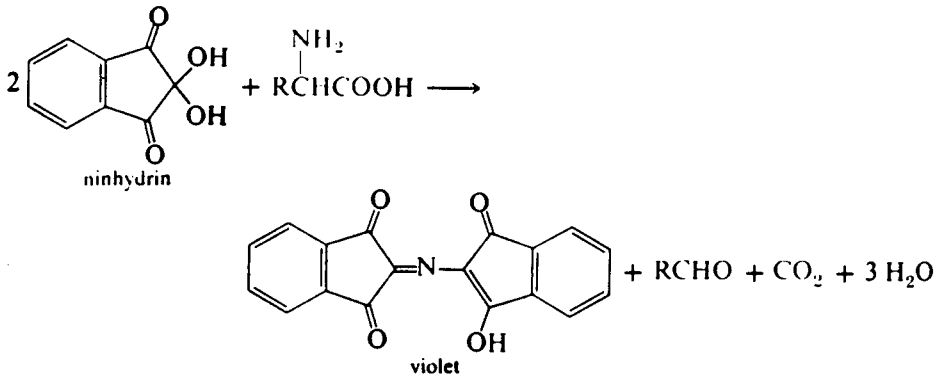


Figure 5.5 Ninhydrin Reaction

Source: Streitwieser, A.; Heathcock, C.H. *Introduction to Organic Chemistry*, 3rd ed.; Macmillan: New York; 1984, p 940.

Capping acylates any unreacted N^{α}-groups with acetylimidizole to prevent them from reacting in the subsequent coupling cycles (see Figure 5.4). Acetylimidizole was used because it is more efficient than the original method using acetic anhydride (Barany and Merrifield, 1979). Capping with acetylimidizole selectively blocks the unreacted amino groups without causing side chain modification or racemization of the peptide (MilliGen/Biosearch, Model 9500 Operator's Guide). Although the standard synthesis protocol averages a 99% coupling efficiency, acetylation of deletion peptides makes the final purification of the desired product easier. The acylated peptides have differing solubility or charge from the desired product, which simplifies HPLC purification (Barany and Merrifield, 1979).

The solid phase synthesis gave a range of 56.0% - 87.3% of expected weight gain. The average synthesis recovery was 75% ± 11%. The difference in yields could be accounted for by sequence variation of the peptide. For the L-amino acid substituted analogues [Asn^2]-dynorphin A-(1-13)NH_2 gave the lowest recovery while [Lys^2]- and [Ser^2]-dynorphin A-(1-13)NH_2 gave the highest.

5.1.3 Cleavage

The use of anhydrous HF is a popular cleavage reagent because it removes almost all benzyl-based protecting groups (see Figure 5.6). HF is a very strong acid which can cause serious alkylation and acylation side

reactions damaging the peptide structure during cleavage. The damage to the peptide is due to the generation of carbocations (Tam and Merrifield, 1987).

Asn is susceptible to side chain hydrolysis during HF cleavage while the major side reaction with Tyr is the alkylation of the aromatic ring. Both of these reactions are irreversible. Ser can exhibit the problem of intramolecular migration from the α-amine acyl group to the hydroxyl group, and the sulfhydryl of Met is subject to oxidation during HF cleavage. The side reaction of Ser is reversible with treatment by an aqueous base and the sulfhydryl oxidation of Met can be reversed by reduction (Stewart and Young, 1984).

Anisole was used in the cleavage reaction as a scavenger to absorb the carbocations generated and protect Tyr from alkylation. Additionally, the potential for oxidation of Met and D-Met was decreased by running the cleavage reaction under nitrogen and keeping the exposure to air at a minimum.

The yield of all analogue cleavages ranged from 48.6% for D-Asn^2 dynorphin A-(1-13)NH_2 to 110.6% for D-Lys^2 dynorphin A-(1-13)NH_2. The average recovery from cleavage was 77% ± 19%. The cleavage yields for D-Asn^2 and D-Lys^2 can be explained by unequal splitting of the precursor resin during synthesis. The cleavage yields for the L-isomers averaged 70% ± 14 and the yields for the D-isomers averaged 78% ± 20%. Although the D-Leu^2 and D-Ser^2 were not dried on the vacuum pump after synthesis and prior to

cleavage, they had some of the highest cleavage yields. Of the groups of peptides cleaved simultaneously, there seems to be a correlation of lower yields for Met^2 and Leu^2 that were cleaved together. This could be explained by an unidentified problem during the cleavage or subsequent work-up.

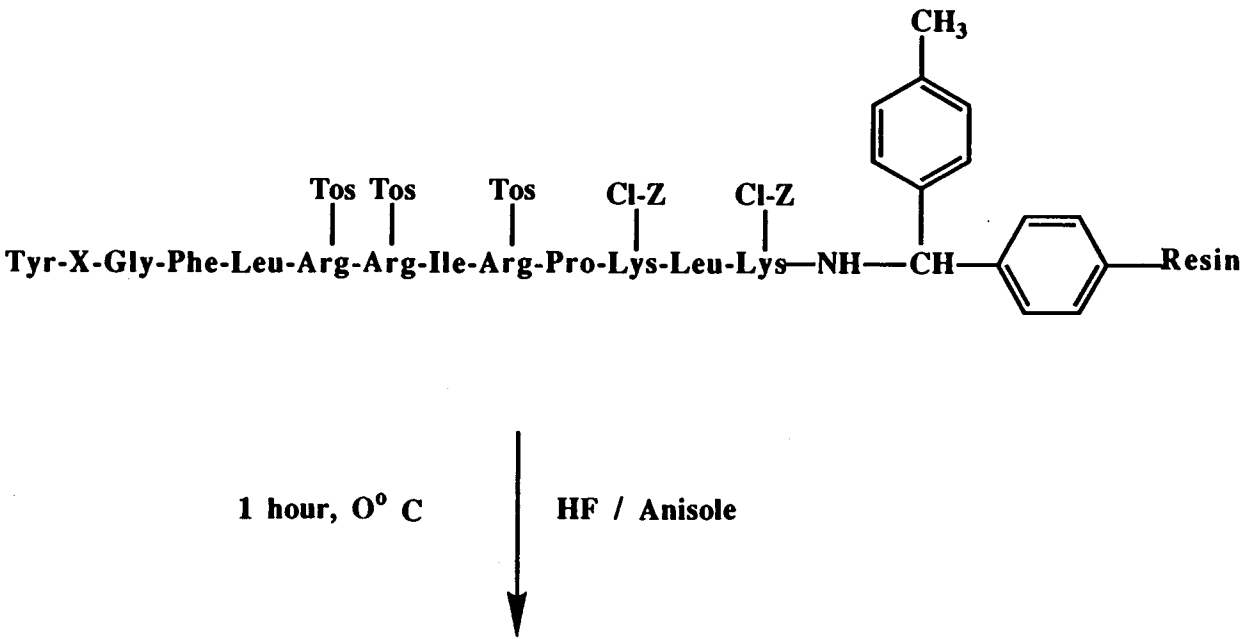


Figure 5.6 HF Cleavage

5.1.4 Purification

During the G-10 purification, weight loss resulted from the removal of contaminants such as resin debris, impurities and salt. The range of recovery from the G-10 column was 38.8% for [D-Asn^2]- to 83.1% for [D-Lys^2]-dynorphin A-(1-13)NH_2. The average recovery from G-10 column purification was 61% ± 15%. There was not any notable procedural error to account for the wide range of recovery.

HPLC is used in peptide purification because it has the distinct advantage over other forms of liquid chromatography in that it can permit complete separation of compounds with very similar properties. Other advantages of HPLC include: speed, reproducible results, a reusable column, automation, and adaptability to large scale.

The solvent system used for reverse phase purification of most peptides in the literature is HPLC grade H_2O + TFA for the weaker eluting solvent and HPLC grade AcCN + TFA for the stronger eluting solvent. TFA is used in the solvents to obtain a sharper peak. The exact mechanism of action is unknown, but it may be migrating with the peptide as an ion pairing reagent or it may simply be adding on the column as an ion exchanger (Bennett et al., 1979). Most of the peptide loss was during preparative HPLC.

The yield of pure peptide ranged from 9.8% for [D-Ser^2]- to 39.7% for [Leu^2]-dynorphin A-(1-13)NH_2. The average recovery from HPLC was 27%

± 11% which is significantly lower than at synthesis, cleavage, or G-10 purification.

A possible explanation for the range of recovery after HPLC may have been the difference between filtration or centrifugation of the sample before preparative purification. Dynorphin A-(1-13)NH_2 samples were filtered before purification as were the [D-Asn^2]-, [D-Leu^2]-, [D-Ser^2]- and [D-Lys^2]-analogues. These analogues averaged a recovery of 20.9% ± 13.9. The [Leu^2]-, [Ser^2]-, [Asn^2]-, [Lys^2]-, [Met^2]- and [D-Met^2]dynorphin A-(1-13)NH_2 analogues were centrifuged prior to purification. These analogues averaged a recovery of 30.5% ± 7.6. The analogues that were centrifuged instead of filtered before purification had about a 10% higher average recovery than did their filtered counterparts. The centrifuged samples also had a 30% smaller variance. This difference might be explained in that the filtered samples tended to foam causing a procedural loss before purification.

Increasing the length of the purification gradient also generally increased the final yield. Another explanation for the large loss during HPLC purification might be extraction of nonpeptide material not removed by the G-10 column or the adherence of the basic peptide to the HPLC column. Interestingly, it was observed that generally on the HPLC column the majority of the impurities eluted before the main peptide peak suggesting that the impurities were more polar than the desired peptide.

5.2 Analysis of Purity

The results from FAB-MS confirmed the molecular weight for each of the peptides synthesized. FAB-MS is an especially useful tool for determining the molecular weights for polar compounds like peptides. The amino acid analysis confirmed the amino acid composition of each peptide synthesized.

The analytical HPLC on the purified peptide allows quantification of the purity of the peptide and allows comparison of retention times or retention volumes from one peptide to another. With the peptides analyzed in this study, the use of a gradient method was more effective in giving reproducible retention times than the isocratic method.

5.3 Pharmacology

5.3.1 Guinea Pig Ileum Assay

The peptides were evaluated for opioid activity in the electrically stimulated muscle of the guinea pig ileum (GPI) (Ward et al., 1982) by Dr. Gary DeLander. Activity was determined in the presence of a mixture of peptidase inhibitors (10 μM bestatin, 10 μM captopril, 0.3 μM thiorphan, and 2 nM Leu-Leu) (McKnight et al., 1983). Agonist activity was measured as the percent inhibition of the twitch and IC_{50} values determined from cumulative does-response curves (see Table 5.1).

Table 5.1 Opioid Activity of Dynorphin A-(1-13)NH_2 Analogues in the Guinea Pig Ileum

Analogue	IC_{50} (nM)[1]	Relative Potency (%)
Gly	0.252	100
Asn	739	<0.1
D-Asn	2.12	0.4
Ser	357	<0.1
D-Ser	2.59	7.8
Met	80.5	0.2
D-Met	3.20	7.6
Leu	10,900	<0.1
D-Leu	31.9	<0.1
Lys	6,710	<0.1
D-Lys	99.5	<0.1

[1] ± standard deviation, n = 3

5.3.2 Radioligand Binding Assay in Guinea Pig Cerebellum

The peptides were examined, by Dr. Thomas Murray, for their ability to inhibit the binding of [^{3}H]bremazocine to guinea pig cerebellar membranes, a tissue in which the kappa opioid receptors account for > 80% of the opioid receptors (Robson et al., 1984). Guinea pig cerebellar membranes were prepared and [^{3}H]bremazocine binding assays performed as previously described by Corbett et al. (Corbett et al., 1982) and Robson et al. (Robson et al., 1984). The binding assays were carried out at 0°C for 240 minutes (see Table 5.2).

Table 5.2 Radioligand Binding of Dynorphin A-(1-13)NH_2 Analogues

Radioligand Binding Assays

Analogue	IC_{50} (nM)[1]		Relative Potency (%)
Gly	0.281	± 0.106	100
Asn	196.5	±47.5	<0.1
D-Asn	0.465	± 0.056	15.3
Ser	72.7	±13.8	0.1
D-Ser	0.699	± 0.020	10.2
Met	29.1	± 7.8	0.3
D-Met	4.89	± 0.80	1.4
Leu	31.3	± 9.8	0.4
D-Leu	22.0	± 2.4	0.3
Lys	229.0	±35.1	<0.1
D-Lys	512.9	±73.8	<0.1

[1] ± standard error

CHAPTER 6

CONCLUSIONS

Amino acid substitution at the 2 position of dynorphin A-(1-13) amide produced marked differences in both opioid activity and kappa receptor binding. The IC_{50} values for the analogues varied over four orders of magnitude in both pharmacological assays.

The parent compound, dynorphin A-(1-13) amide, which contains Gly in the 2 position, was the most potent compound tested. The [D-Asn^2]-, [D-Ser^2]-, and [D-Met^2]dynorphin A-(1-13) amide analogues were the most potent of the synthetic peptides prepared.

For Asn, Met and Ser, the analogues containing the D-isomer in the 2-position were much more potent than the analogues containing the L-amino acid. These results parallel those previously reported for Ala and D-Ala^2 dynorphin A-(1-13) analogues (Lemaire et al., 1986; Chavkin and Goldstein, 1981). The Ala^2 analogue retained only a 0.6% relative potency in the guinea pig ileum (Lemaire et al., 1986) while the D-Ala^2 analogue retained 17% relative potency (Chavkin and Goldstein, 1981). The polar Asn analogue retained less than 0.1% of biological activity in the guinea pig ileum, while the nonpolar Met^2 analogue retained a smattering of biological activity (0.2%). On the other hand, the D-isomers of these three analogues retained

significant biological activity. The D-Asn^2 analogue retained 10.4% biological activity and the D-Ser^2 and D-Met^2 analogues retained 7.8% and 7.6% of the GPI activity, respectively.

The least potent of the peptides synthesized were the Lys^2 and D-Lys^{2}dynorphin A-(1-13)NH_2 analogues. A basic residue in the "message" sequence rather than the "address" sequence of the peptide markedly decreases both opioid activity and receptor binding.

In contrast to [Ala^2]dynorphin A-(1-13) (Lemaire et al., 1986), which showed decreased agonist activity while retaining opioid receptor binding affinity, most of the synthetic opioid peptides prepared in this study generally paralleled the opioid binding affinity in the guinea pig cerebellum.

The pharmacological data suggests that changing the conformation of the Gly^2 position by substitution of a polar D-amino acid retains a portion of the opioid activity and receptor binding. These results do not support the hypothesis that α-helical conformation promotes biological activity or kappa receptor binding.

The results of this study lay the ground work for further structure-activity studies of dynorphin A using modifications in the Gly^2 position to make conformationally constrained analogues and in further testing of Gly^2-substituted analogues for potential antagonist activity.

PART TWO

SYNTHESIS OF A PROTECTED PEPTIDE AMIDE

CHAPTER 7

LITERATURE REVIEW

7.1 Fmoc Chemistry

The classical Boc chemistry introduced by Merrifield uses repetitive TFA treatments to remove the temporary amine protecting groups. Repetitive TFA treatments can cause side reactions and premature cleavage of the growing peptide from the support (Paivinen et al., 1987; Atherton et al., 1978). The side chain protecting groups used with the Boc strategy are benzyl derivatives and require a strong acid, such as HF or HBr/HOAc, for removal. The linkage between the peptide and the resin also generally requires a very strong acid, such as HF, for cleavage. This final HF cleavage is frequently destructive of peptide bonds as well as causing other side reactions (Feinberg and Merrifield, 1975). The use of HF requires a special apparatus to protect the chemist and equipment from the poisonous and highly corrosive effects of this reagent. Further, this use of strong acid does not allow selective

cleavage of resin linkage and side chain deprotection (Atherton and Sheppard, 1987).

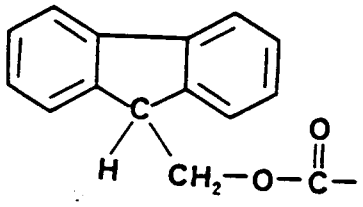
These disadvantages of the Boc chemical protocol prompted the development of the Fmoc (9-fluorenylmethoxycarbonyl) protecting group (Carpino and Han, 1970, 1972) (see Figure 7.1) and its later adaptation to solid phase methodology (Chang and Meienhofer, 1978; Atherton et al., 1978). The Fmoc protecting group is base-labile and thus eliminates the need for repetitive acidolysis in removal of the temporary amine protecting group. This base-labile group can be rapidly removed by a secondary amine such as piperidine. The use of a base-labile temporary amine protecting group allows the restructuring of side chain protection and resin linkage. The Fmoc strategy uses acid-sensitive protecting groups such as the tBu derivatives which can be removed by TFA. The use of TFA-sensitive side chain protecting groups eliminate damage, danger and expense associated with the use of HF.

This scheme combines the base-labile N^{α}-amine protecting group with the acid-labile t-butyl groups (tBu) for Tyr, Ser and Thr and the t-butyl ester (OtBu) for Glu and Asp. Gln and Asn may be protected with either 2,4,6-trimethoxybenzyl (Tmob) or 4,4,-dimethoxybenzhydrol (Mbh) while the Boc group is for the side chain protection of Lys. Arg is routinely protected with either 4-methoxy-2,3,6-trimethylbenzenesulphonyl (Mtr) or 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) protecting group (see Figure 7.1) (Riniker and Hartman, 1989; Ramage et al., 1988). Cys may be protected with

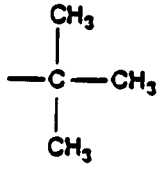
trityl (Trt), which is acid-labile, or with the acetamidomethyl (Acm) group, which requires removal by oxidation with iodine or mercuric salts.

Further, the potential for differences in acid lability of side chain protecting groups and the resin linker allows a pseudo-orthogonality for preferential deprotection of side chain protecting groups and cleavage of the peptide-resin bond (Atherton and Sheppard, 1987). Orthogonality is defined as a system in which independent classes of protecting groups can be removed in any order in the presence of all other classes (Barany and Merrifield, 1977).

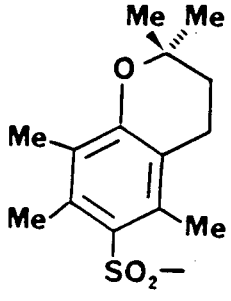
Fmoc



tBu



Pmc



Mtr

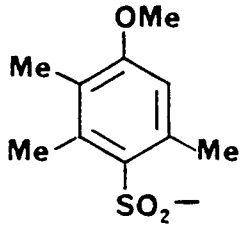


Figure 7.1 Structure of Protecting Groups Used in Fmoc Chemistry

Along with the development of the Fmoc protection strategy new choices of coupling methods has been made available. Merrifield gave thought to using active esters in the Boc solid phase protocol but the idea was not incorporated into the system because of success with the carbodiimide coupling method (Bodansky and Bednarek, 1989).

The coupling methods currently used in Fmoc chemistry are either DIPCDI, (benzotriazolyloxotris(dimethylamino)-phosphonium hexafluorophosphate (BOP), or active esters.

The use of the pentafluorophenyl (OPfp) active ester in the Fmoc solid phase synthesis was developed in the mid-eighties (Kisfaludy and Schön, 1983; Atherton and Sheppard, 1985). The OPfp ester is still the most ideal of the active ester available for use in Fmoc solid phase synthesis (Hudson, 1990). The OPfp esters react more slowly than the symmetrical anhydrides but the reaction time is increased using the HOBt coupling additive (Atherton et al., 1988).

The BOP protocol employs the use of Castro's reagent (Castro et al., 1975) in conjuction with HOBt and requires N-methylmorpholine (NMM) as an activating base. The BOP mechanism is uncertain and may take one of two routes. The incoming amino acid in the presence of BOP and NMM forms the acyloxyphosphomium salt which reacts with HOBt to from an hydroxybenzotriazole active ester which couples the residue (see Figure 7.2). In the other route the amino acid in the presence of BOP and NMM can

form the symmetrical anhydride and react with HOBt to form the hydroxybenzotriazole active ester which goes to product (Hudson, 1988). Recently Knorr and coworkers have data suggesting the use of the related tetramethyluronium hexafluorophosphate derivative in place of BOP with the continuous-flow instrument (Knorr et al., 1989).

The Biosearch 9500 instrument is designed for use with the DIPCDI protocol (see Section 2.8.1). Coupling may be achieved using an active ester, but must be done manually. This particular instrument was not designed for use with the BOP protocol.

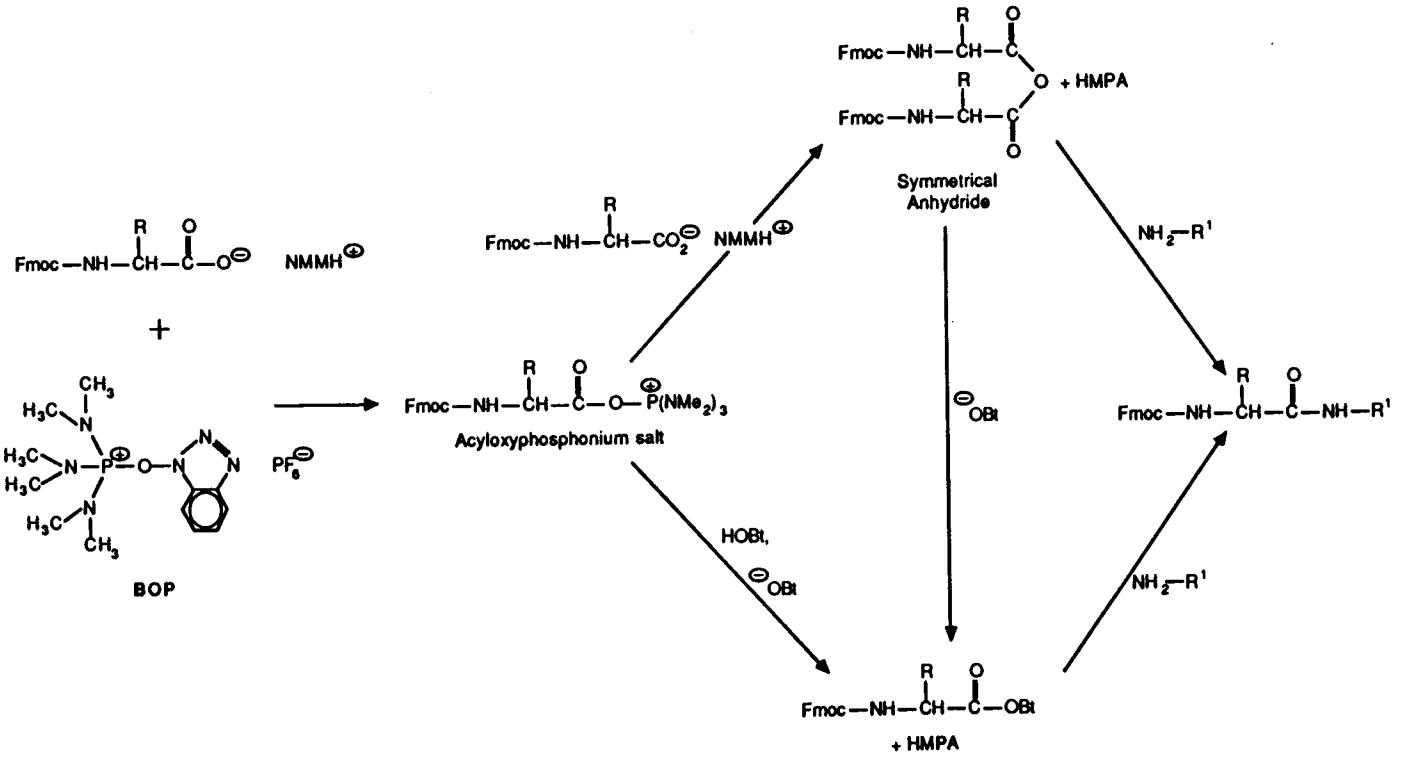


Figure 7.2 BOP Activation

Source: Biosearch Technical Bulletin No. 9000-03

7.2 Resins used in Fmoc Chemistry

The resins used in solid phase peptide synthesis are generally either polystyrene- or polyamide-based. The polystyrene resins are crosslinked with 1% divinylbenzene for optimum swelling and stability. The polystyrene beads swell in methylene chloride to approximately five times their dry volume (Fields and Noble, 1990).

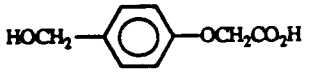
Another type of resin designed for use in the batch-type synthesis is the "gel" resin which has a polyamide-based support. This support is a polymer of a crosslinked dimethylacrylamide, functionalized with acryloylsarcosine methyl ester to provide attachment sites and obtain optimum solvation properties (Arshady et al., 1979).

Generally, the resins used with a continuous-flow instrument, in which the resin is continually subjected to solvent flow in a column-type format, are made of a composite Kieselguhr/polyamide matrix (Fields and Noble, 1990).

7.2.1 Peptide Acids

The synthesis of peptide acids on a batch-type instrument may be accomplished by the use of either a polyacrylamide gel resin or a polystyrene resin. The acid-labile 4-hydroxymethylphenoxyacetic acid linker (Linker A) (see Figure 7.3) has been attached to both a polydimethacrylamide gel resin (Pepsyn A) and the polystyrene MBHA resin (PAC resin). A peptide acid

may be prepared on a continuous-flow instrument using the rigid Kieselguhr composite resin (Pepsyn KA) which also has linker A attached.



4-hydroxymethylphenoxyacetic acid

Figure 7.3 Fmoc Resin Linkers for Peptide Acids

Source: Fields, G.B.; Noble, R.L. *Int. J. Peptide Protein Res.* **1990**, *35*, p 166.

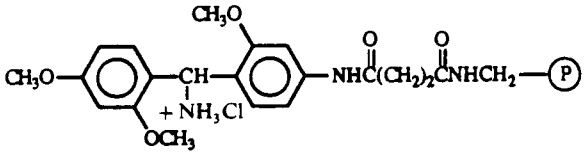
7.2.2 Peptide Amides

The synthesis of a peptide amide has been difficult using the Fmoc protocol on a polystyrene resin (see Figure 7.4 for structures of resins and linkers). This is because there had been no equivalent of the acid-labile MBHA resin used with Boc chemistry. Peptide amides were originally produced on a p-nitrobenzhydrylamine resin with cleavage by catalytic hydrogenation (Colombo, 1981).

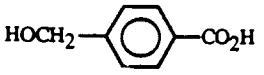
The preparation of a peptide amide may be accomplished on a continuous-flow instrument using the Pepsyn KB Kieselguhr resin, or on a batch-type instrument using a Pepsyn B polyamide resin. Both of these resins have a 4-hydroxymethylbenzoic acid linker (Linker B). This benzyl ester linkage is cleaved by aminolysis to give the amide.

The use of an acid-labile linkage to produce a peptide amide on a polystyrene resin was first achieved with the 2,4-dimethoxybenzhydrylamine resin (Penke and Rivier, 1987). The production of a peptide using an acid-labile linker became more routine with the commercial availability of the PAL resin in 1988. The PAL resin is an MBHA resin, with a 5-(4'-Fmoc-aminomethyl-3',5'-dimethyloxyphenoxy)valeric acid linker (Albericio and Barany, 1987). The 4-succinylamino-2,2',4'-trimethoxybenzhydrylamine (SAMBA) resin (Penke and Nyerges, 1988; Penke et al., 1988) became commercially available in 1989.

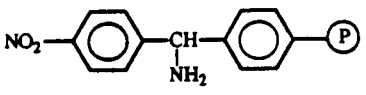
Other acid-labile linkers developed to produce peptide amides are the 4-(4'-methoxybenzhydryl)phenoxyacetic acid (Breipohl et al., 1987) and 3-(amino-4-methoxybenzyl)-4-methoxyphenyl propioinc acid (Funakoshi et al., 1988).



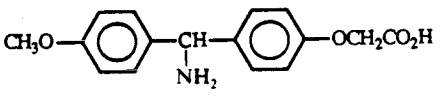
4-succinylamino-2,2′,4′-trimethoxybenzhydrylamine resin



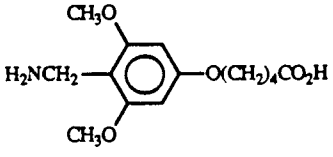
4-hydroxymethylbenzoic acid



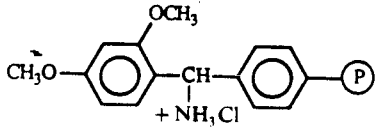
p-nitrobenzhydrylamine resin



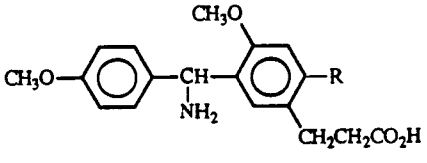
4-(4′-methoxybenzhydryl)-phenoxyacetic acid



5-(4-aminomethyl-3,5-dimethoxyphenoxy)-valeric acid



2,4-dimethoxybenzhydrylamine resin



3-(amino-4-methoxybenzyl)-4-methoxyphenylpropionic acid

Figure 7.4 Fmoc Resin Linkers for Peptide Amides

Source: Fields, G.B.; Noble, R.L. *Int. J. Peptide Protein Res.* **1990**, *35*, p 166.

7.3 Protected Fragments

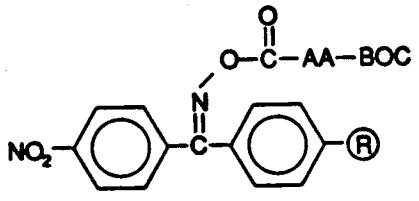
In the past, the protected fragments were prepared by classical solution synthesis because the solid phase procedure, either Boc or Fmoc, had not been adapted efficiently to fragment preparation. The Fmoc chemical protocol has the advantage over the Boc protocol by allowing the preparation of protected peptide fragments using solid phase methodology by an orthogonal protection scheme. The preparation of a protected peptide allows further modifications to be performed on the fragment. Interest in protected fragments generally focused on protected peptide acids. These protected peptide acid fragments were generally used in solution chemistry for segment condensation.

7.3.1 Boc Method

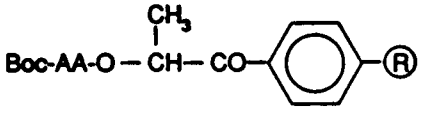
The preparation of protected peptide acid fragments using the Boc protocol has not been feasible until recently. An oxime resin for the preparation of a protected peptide fragment using the Boc chemical protocol has become available in 1990. This resin contains a p-nitrobenzophenone oxime attached to a polystyrene based support (see Figure 7.5). The oxime ester linkage has a limited stability to TFA and may be released prematurely from the resin with lengthy exposure to TFA. To minimize this problem, the TFA deblock concentration should be reduced from 45% to 25% and the

protected fragment limited to 10 residues. This limits the usefulness of this resin with dynorphin fragments. The peptide is cleaved from the resin by nucleophilic attack (Lobl and Maggiora, 1988).

Another resin that allows the preparation of protected peptides using the Boc chemical protocol is the brominated-Wang resin. The Br-Wang resin consists of a brominated α-methylphenacyl linker attached to a polystyrene support (see Figure 7.5). The first amino acid is coupled using the Boc-amino acid cesium salt. The protected peptide is cleaved from the resin using a photochemical reaction chamber via photolytic cleavage (Wang, 1976).



oxime resin



Br-Wang resin

Figure 7.5 Resins for Protected Fragments Using Boc Protocol

7.3.2 Fmoc Method

A variety of peptide-to-resin linkers permits eventual cleavage of peptides as protected acid fragments using Fmoc chemistry (see Figure 7.6 for structures). The synthesis of a protected peptide acid on a continuous-flow instrument is currently only possible on the acid-labile Kieselguhr (Pepsyn KH) (Dryland and Sheppard, 1986) resin which has a 4-hydroxymethyl-3-methoxyphenoxyacetic acid linker (Linker H).

A acid-labile polyamide resin (Pepsyn H) (Arshady et al., 1979) may be used on a batch-type instrument, which also uses linker H. Other linkers have been designed for use with the gel resin. They include a 3-nitro-4-hydroxymethylbenzoic acid linker, which is cleaved by photolysis at 350 nm in 80% toluene/20% tetrafluoroethanol (TFE) (Kneib-Cordonier et al., 1990), or the 2,4-dialkoxybenzyl alcohol linker (Sheppard and Williams, 1982) which is cleaved with TFA.

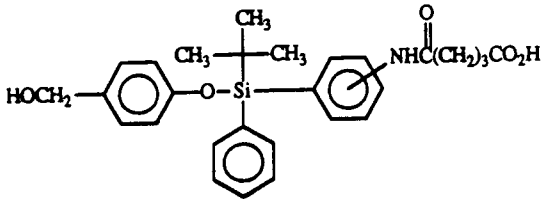
In 1987, Rink introduced a mild acid-labile polystyrene resin for the synthesis of protected peptide acids on a batch-type instrument. This 4-(2',4'-dimethoxyphenylhydroxymethyl)-phenoxymethyl resin has the advantage over the gel resin of being polystyrene based and using only 0.2% TFA in DCM for cleavage of the resin-peptide linkage (Rink, 1987).

The Sasrin resin is also a polystyrene resin that has become commercially available which also may be used to prepare a protected peptide

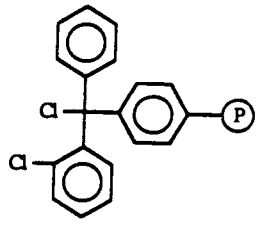
acid. It is a 2-methoxy-4-alkoxybenzyl alcohol resin which is also cleaved with a weak TFA solution (Mergler and co-workers, 1988a,b).

The Hycram resin is a polystyrene resin that allows the production of protected peptide acids via ether Boc or Fmoc chemistry which became commercially available in 1990. This hydroxy-crotonyl-aminomethyl resin is stable to both acids and bases and is cleaved under neutral conditions at room temperature. This resin uses a allylic crotonyl group attached to the support. The peptide is cleaved from the resin with an allyl acceptor such as tetrakis (triphenylphosphino) palladium (O) for Boc synthesized peptides and by dimedone for peptides synthesized by Fmoc (Kunz and Dombo, 1988).

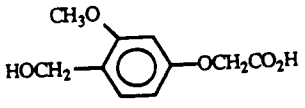
Other possibilities include a 2-chlorotrityl-chloride resin, which is cleaved by 10% AcOH/10% TFE/80% DCM (Barlos et al., 1989). More novel linkers include those containing silicon such as (3 or 4)-[[[(4-hydroxymethyl)phenoxy-t-butyl-phenyl]siyl]phenyl]pentanedoic acid, monoamide or 3-(4-hydroxymethylphenyl)-3-trimethylsilylpropionic acid which can be attached to an aminomethyl resin (Mullen and Barany, 1987; Ramage et al., 1987) or to a MBHA resin (Mullen and Barany, 1988). These silicon containing linkers are cleaved by tetrabutylammonium fluoride (TBAF).



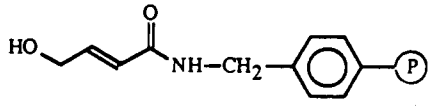
(3 or 4)-[[[(4-hydroxymethyl)phenoxy-t-butyl-phenyl]silyl]phenyl] pentanedioic acid, monoamide



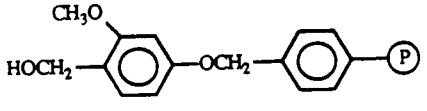
2-chlorotrityl chloride resin



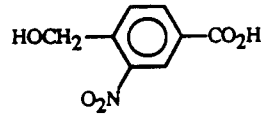
2-methoxy-4-hydroxymethylphenoxyacetic acid



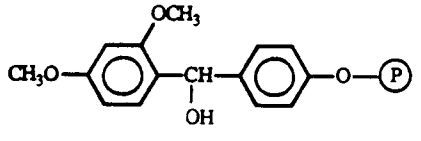
hydroxy-crotonyl-aminomethyl resin



2-methoxy-4-alkoxybenzyl alcohol resin



3-nitro-4-hydroxymethylbenzoic acid



4-(2',4'-dimethoxyphenylhydroxymethyl)-phenoxymethyl resin

Figure 7.6 Fmoc Resins for Protected Peptide Acids

Source: Fields, G.B.; Noble, R.L. *Int. J. Peptide Protein Res.* **1990**, *35*, p 166.

CHAPTER 8

RATIONALE AND OBJECTIVES

8.1 Rationale

As discussed earlier, a current goal in analgesic research is to separate the desired analgesic effect from undesired effects such as addiction. Distinct receptor types may play different roles in producing either the desirable or the undesirable effects associated with narcotic analgesia. It is difficult to sort out the physiological roles of one specific receptor type when multiple receptors are present. Thus, a ligand that could selectively block one receptor type from the others would be a useful pharmacological tool.

Affinity labels are ligands which bind irreversibly to a receptor. These affinity labeled compounds can be used to block selected receptor populations in tissues which contain multiple receptor types so that the remaining receptors can be studied independently (Takemori and Portoghese, 1985). Knowledge gained from such studies could ultimately lead to a better understanding of the function of different opioid receptors.

Protected peptide fragments are useful precursors because they can be further modified to incorporate reactive functionalities such as affinity labels, radioactive tracers or molecules such as biotin for receptor investigation.

Protected dynorphin analogues are of particular interest because they can be modified to incorporate reactive functionalities (affinity labels) to study the kappa opioid receptor.

8.2 Objectives

The ultimate goal of this project is to prepare an affinity label based on Phe^4- modified [D-Pro^{10}]dynorphin A-(1-11)NH_2.

A peptide amide was chosen for preparation because of the advantage of increased metabolic stability over the endogenous peptide acid (for further discussion of the metabolic stability of the peptide see section 2.6). A C-terminal amide was also chosen because it decreased the possibility for side reactions during synthesis. The [Pro^{10}]dynorphin A-(1-11)NH_2 analogue was chosen because it has been shown to have a higher kappa receptor selectivity and increased metabolic stability due to the replacement of Pro with its D-isomer (Gairin et al., 1988). The Phe^4 position of the "message" sequence of the dynorphin analogue was chosen for the incorporation of an affinity label because modifications in that position are well tolerated (Schiller, 1982).

The approach used to prepare the affinity label was to synthesize the protected peptide amide precursor (BocTyr(tBu)-Gly-Gly-Phe(NO_2)-Leu-Arg(Pmc)-Arg(Pmc)-Ile-Arg(Pmc)-DPro-Lys(Boc)NH_2), cleave it from the

resin and incorporate the affinity label into the protected peptide in solution and deprotect the peptide.

The first step in this project was to develop a method for the preparation of a protected peptide amide. This was necessary since there were no established method for production on a batch-type instrument for this purpose. The preparation of a protected peptide amide on a polystyrene-based resin for use with a batch-type automated instrument had not to my knowledge been achieved prior to this work.

The preparation of a protected peptide amide model compound (BocTyr-Gly-Gly-Phe-Leu-Arg(Mtr)NH_2 or BocTyr-Gly-Gly-Phe-Leu-Arg(Pmc)NH_2) was attempted using three resins. This model compound was chosen because it was the smallest fragment of the goal peptide that contained the troublesome Arg residue. The first resin investigated was a polyamide resin, the second was a polystyrene resin (cleavage conditions modified to produce a protected peptide amide) and the third a polystyrene resin developed in this laboratory for this project.

CHAPTER 9

EXPERIMENTAL

9.1 Instrumentation and Reagents

The instrumentation used was the same as described in Chapter 4; also used was a Beckman DB spectophotometer. Sources of additional reagents are: BocAla, FmocAlaOPfp, Fmoc-protected amino acids, 4-hydroxymethylbenzoic acid pentafluorophenyl ester (Pepsyn B Linker-OPfp), 1,4-dimethylaminopyridine (DMAP) and Pepsyn gel resin (Milligen/Biosearch, Novato, CA); 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy "Rink" Resin (Calbiochem, San Diego, CA); FmocArg(Pmc) (Bachem, Torrence, CA or Milligen/Biosearch, Novato, CA); DMF and MeOH (Burdick and Jackson); sodium bicarbonate, sodium chloride, sodium sulfate (Baker); thioanisole, 1,2-ethanedithiol, DCC, ethylenediamine and piperidine (Aldrich); iPrOH and toluene (Merck); NH_3 (Matheson).

9.2 Pepsyn Gel Resin

9.2.1 Functionalization of Gel Resin

The resin was functionalized according to a modification of MilliGen Technical Note 4.20 (see also Arshady et al., 1981). The Pepsyn gel resin (1.0128 g; 0.3 meq/g) was reacted with ethylenediamine (35 mL) overnight at room temperature. The resin was washed 10 × with about 20 mL DMF and neutralized 2 × with about 20 mL of 10% DIPEA in DMF. The resin was washed 5 × with 20 mL DMF. The resin tested very positive by ninhydrin.

FmocAlaOPfp (0.09 M; 1.00g dissolved in 20 mL DMF) was coupled to the ethylenediamine spacer of the gel resin for 60 min and washed 5 × with 20 mL DMF. The resin tested slightly ninhydrin positive after the addition of the FmocAla. The Fmoc protecting group was removing by washing the resin once with 20mL of 20% piperidine in DMF for 3 min, and a second time for 7 min. The resin was washed 10 × with 20 mL DMF and tested ninhydrin positive. The Pepsyn Linker B-OPfp active ester (0.09 M; 576 mg dissolved in 10 mL DMF) was coupled to the resin for 60 min and washed 10 × with 20 mL DMF. The resin tested very slightly ninhydrin positive.

9.2.2 Esterification of the Gel Resin

The resin was esterified according to MilliGen Technical Note 4.30 (see also Arshady et al., 1981). A 12-fold excess of FmocArg(Mtr) (3.60 mmoles; 0.01 M; 2.21 g in 30 mL DCM) was reacted with DCC (1.8 mmoles; 377 mg) at room temperature for 10 min to form the symmetrical anhydride. The DCU byproduct was removed by filtration and the filtrate evaporated *in vacuo* to give the solid symmetrical anhydride. The FmocArg(Mtr) symmetrical anhydride was dissolved in 20 mL of DMF, refiltered and added to the resin. A catalytic amount of DMAP (0.3 mmol; 37 mg) was dissolved in 5 mL of DMF and also added to the resin mixture. The resin mixture was reacted at room temperature for 107 min and after filtering, the resin was washed with DMF (4 × 20 mL), DCM (4 × 20 mL), MeOH (2 × 20 mL) and DCM (2 × 20 mL). After washing, the resin was a gelatinous solid maintaining the shape of the reaction vessel.

9.2.3 Quantitative Resin Loading

The esterified resin was subjected to quantitative Fmoc analysis to determine the loading of the FmocArg residue to the functionalized resin (MilliGen Technical Note 3.10). A sample of approximately 5 mg of the resin was accurately weighted, and transferred to a 25 mL volumetric flask to which 400 μL piperidine and 400 μL DCM were added. The flask was capped and the deprotection reaction proceeded for 30 min. To the volumetric flask 1.6

mL MeOH was added and diluted to volume with DCM. A reference standard was made adding 400 μL piperidine, 400 μL DCM and 1.6 mL MeOH to a 25 mL volumetric flask and diluting to volume with DCM. The absorbance was measured at 301 nm. This experiment was run in duplicate. The data from the quantitative Fmoc showed a substitution of 0.16 mmole/g (65.5%) at Arg.

After Fmoc removal (see Section 9.2.4 for the Fmoc removal procedure) from FmocArg(Mtr) the resin was sent for quantitative amino acid analysis to determine the loading of both the Ala and Arg residues. The amino acid analysis showed a loading of 0.25 mmole/g (100.0%) at Ala and 0.11 mmole/g (47.8%) at Arg.

9.2.4 Attempted Synthesis of Model Compound on Gel Resin

The model peptide, BocTyr-Gly-Gly-Phe-Leu-Arg(Mtr)NH_2, was synthesized on the functionalized gel resin using the Fmoc protocol. The functionalized resin was swollen in DCM/DMF (1:1) for 10 minutes prior to the beginning of the synthesis. The solid phase synthesis cycle commenced with 4 × 5 mL each DCM/DMF (1:1) washes of the resin and the removal of the Fmoc temporary amine protecting group from FmocArg(Mtr) using a 30% piperidine, 35% toluene and 35% DMF solution; the resin was deblocked for three minutes, then for seven minutes with the piperidine solution followed by 10 × 5 mL washes of DCM/DMF (1:1). The Fmoc-

amino acids were coupled to the growing chain by reacting a 6.67-fold excess (5 mL of a 0.4M Fmoc amino acid in DMF) with 5 mL of 0.4M DIPCDI plus 1 equivalent of HOBt in DCM for two hours, followed by 4 × 5 mL each DCM/DMF (1:1) washes. Ninhydrin tests were performed at each coupling and were negative. After the final amino acid was coupled to the resin and the Fmoc removed the resin was washed with 10 × 5 mL of DCM/DMF (1:1), 7 × 5 mL of DCM, and 4 × 5 mL of MeOH. This 19-hour synthesis required 4 days to complete due to the failure at each step of the reaction vessel to empty properly through the gelatinous resin. The failure of the reaction vessel to empty required constant manual manipulation.

The synthesis used 1.0128 g (before functionalization) of resin (0.3 mmole/g before functionalization) and had a theoretical final weight of 1.3887 g. The final weight of the resin and peptide was 388.5 mg for a recovery of only 28.0%.

9.2.5 Cleavage of Model Compound from Gel Resin

The gel resin (388.5 mg) was cleaved by aminolysis (see Stewart & Young. p.91). The resin was suspended in a heavy-wall pressure bottle containing approximately 10 mL MeOH with a magnetic stirrer in the hood. The stirring solution was cooled in an ice bath for 5 minutes then saturated with NH_3 by slowly bubbling anhydrous NH_3 into the vessel, excluding moisture by use of a KOH trap, for 20 minutes. The capped reaction vessel

was stoppered, wired closed and allowed to warm to room temperature. The reaction proceeded at room temperature for 3 hours.

The pressure bottle was cooled again in an ice bath and the cap removed. The bottle was warmed to room temperature and stirred vigorously to allow the NH_3 to escape into the hood. The resin was filtered and washed with MeOH (3 × 10 mL each). The combined MeOH and washes were combined and evaporated to give 52.4 mg (16.9%) of the crude protected peptide.

9.3 Rink Resin

9.3.1 Synthesis of Model on Rink Resin

The model peptide, BocTyr-Gly-Gly-Phe-Leu-Arg(Pmc)NH_2, was also synthesized using the Fmoc chemical protocol on the "Rink" resin (1.0 g scale; 0.57 mmole/g). The polystyrene-based resin was swollen in DCM/DMF (1:1) for at least 10 minutes prior to the beginning of the synthesis. The Fmoc protecting group was removed and the FmocArg(Pmc) and subsequent amino acids were attached using the DIPCDI coupling method as described in Section 2.8.4. The coupling additive HOBt was not used in this synthesis.

The synthesis used 0.9837 g resin and had a theoretical weight after synthesis of 1.4541 g. The weight of the resin after synthesis was 1.4534 g for a weight gain of 99.9% of the expected value.

9.3.2 Cleavage of Model from Rink

The resin (314.1 mg) was cleaved using approximately 6 mL of a 2% TFA in DCM solution for four hours at room temperature. The resin was filtered from the peptide solution and washed with 3 × 10 mL of the TFA solution. The filtrate was washed with 5% $NaHCO_3$ (3 × 20 mL), distilled water (3 × 20 mL), saturated NaCl (3 × 20 mL); and dried with Na_2SO_4. After filtering the sodium sulfate the DCM was evaporated and the solid dried *in vacuo* to give 13.2 mg crude peptide (10.1%).

Another aliquot of resin (145.8 mg) was cleaved using approximately 6 mL of modified Reagent R (87% TFA, 5% thioanisole, 3% ethanedithiol, 5% water) for 6 hours at room temperature. The resin was filtered through glass wool, and the peptide precipitated in cold ether (approximately 250 mL) and refrigerated overnight. The peptide was filtered from the ether, washed with ether 3 × 20 mL, and dried *in vacuo* to give 23.4 mg of crude peptide (28.5%).

9.3.3 Synthesis of Precursor on Rink Resin

The precursor peptide, BocTyr-Gly-Gly-Phe(pNO_2)-Leu-Arg(Pmc)-Arg(Pmc)-Ile-Arg(Pmc)-DPro-Lys(Boc)NH_2, was also synthesized using the Fmoc chemical protocol on the "Rink" resin (1.0 g scale; 0.57 mmole/g) as described in section 9.3.1.

The synthesis used 1.0082 g resin and had a theoretical weight after synthesis of 2.2949 g. The weight of the resin after synthesis was 2.2516 g for a weight gain of 98.1% of the expected value.

9.3.4 Cleavage of Precursor on Rink Resin

When the precursor (1.1441 g) was cleaved from the resin with 2% TFA in DCM, as described in section 9.3.2., only 7.6 mg of crude peptide (1.1%) was obtained.

9.4 MBHA Resin

9.4.1 Functionalization of the MBHA Resin

The MBHA resin (0.9901 g, 0.50 meq/g) was swollen with DMF/ DCM (1:1) (10 mL) under nitrogen agitation for 15 minutes.

The resin was washed with 3 × 20 mL of DMF/DCM (1:1), then washed with 3 × 20 mL of DCM. The resin was neutralized with 3 × 10 mL of a 10% DIPEA in DCM solution. The resin was washed with 6 × 20 mL of DCM; the resin tested a bright ninhydrin positive. The resin was washed with 3 × 20 mL of DMF/DCM (1:1) after the ninhydrin test.

Boc-Alanine (0.80 mmoles; 1.00 g) and HOBt (3.75 mmoles) were dissolved in 8.6 mL DMF and coupled to the resin with 8 mL of DIPCDI (0.87 M) in DCM. This mixture was agitated for one hour under a gentle

stream of nitrogen at room temperature. The resin was washed with 3 × 20 mL of DMF/DCM (1:1) and with 3 × 20 mL of DCM; the resin tested ninhydrin negative. The resin was neutralized with 3 × 10 mL of a 10% DIPEA in DCM solution, and washed with 6 × 20 mL of DCM and 3 × 20 mL of DMF/DCM (1:1). The resin was coupled again with Boc-Alanine as described above. The resin was washed 3 × 20 mL of DMF/DCM (1:1) and 3 × 20 mL of DCM. The resin tested ninhydrin negative. The resin was neutralized with 3 × 10 mL of a 10% DIPEA in DCM solution, and washed with 3 × 20 mL of DMF/DCM (1:1) and 3 × 20 mL of DMF.

The resin was capped using 25 mL of 1-acetylimidazole (0.30 M) in DMF for 30 min. The resin was washed with DMF, DCM/DMF, and DCM (3 × 10 mL each).

The Boc protecting group was removed using a deblock solution (45% TFA, 2.5% anisole in DCM, 2 × 20 min). The resin was washed with DCM, 10% DIPEA in DCM and DCM (3 × 10 mL each). The ninhydrin test was a positive bright blue. The resin was washed with DCM/DMF (1:1) and DMF (3 × 10 mL each).

The Pepsyn B-OPfp linker (0.50 mmole; 960 mg dissolved in 10 mL DMF) was coupled to the Ala of the resin for one hour. The resin was washed with DMF, DCM/DMF (1:1), and DCM (3 × 10 mL each). The ninhydrin test after the addition of the linker was very weakly positive (yellow-green).

9.4.2 Esterification of the MBHA Resin

Twelve equivalents of FmocArg(Pmc) (5.94 mmoles; 3.94 g) were dissolved in 24 mL of DCM in a flame-dried round bottom flask to obtain a concentration of at least 0.1 M. The FmocArg(Pmc) was reacted with six equivalents of DCC (0.62 g, 3.85 mmole) at room temperature for 10 minutes to form the symmetrical anhydride. The DCU byproduct was removed by filtration and the filtrate evaporated *in vacuo* to form a solid. The FmocArg(Pmc) symmetrical anhydride was dissolved in 15 mL of DMF and added to the functionalized resin. A catalytic amount of DMAP (62.2 mg; 0.50 mmol) was dissolved in 3 mL of DMF and also added to the resin mixture. This mixture was reacted at room temperature for 20.5 hrs.

The resin was washed with DMF (6 × 20 mL), DCM/DMF (1:1), DCM and DMF (3 × 20 mL each) and capped using 25 mL of 1-acetylimidazole (0.30 M) in DMF for 30 min. The resin was again washed with DMF, DCM/DMF (1:1) and DCM (3 × 10 mL each) in preparation for further synthesis.

9.4.3 Quantitative Resin Loading

The functionalized resin was subjected to quantitative amino acid analysis after removal of the Boc protecting group (see Section 4.2.1 for the Boc removal protocol) to determine the loading of Ala to the resin. The amino acid analysis showed an Ala loading of 0.55 mmole/g (129.0%).

The esterified resin was subjected to quantitative amino acid analysis and quantitative Fmoc analysis to determine the loading of the Arg residue, as discussed in Section 9.2.3. The amino acid analysis showed a loading of 0.39 mmole/g (139.9%) of Arg. Quantitative Fmoc analysis also showed a loading of 0.39 mmol/g (107.8%).

9.4.4 Synthesis of Model Compound on MBHA

The model peptide, BocTyr-Gly-Gly-Phe-Leu-Arg(Pmc)NH_2, was synthesized on the functionalized MBHA resin using the Fmoc protocol, as described in Section 9.2.3. This synthesis utilized HOBt (2.2 mmoles per residue) with the amino acids. The resin was tested by ninhydrin at Leu^5 and Gly^2 and found to be negative.

Starting from 0.9901 grams of unfunctionalized resin (0.50 mmole/g) the resin had a final weight of 1.4240 g for 71.3% of the expected weight gain.

9.4.5 Cleavage of Model Compound from MBHA Resin

The MBHA resin (79.0 mg) was cleaved by aminolysis (see Stewart & Young, 1984:91) in a manner similiar to that described in section 9.2.4. The cleavage reaction used iPrOH instead of MeOH and the reaction proceeded for 4 days. After cleavage the resin was filtered and washed with iPrOH (3 × 10 mL). The combined iPrOH and washes were combined and evaporated to give 15.6 mg (52.7%) of the crude protected peptide amide.

9.5 Characterization of Model Peptide

The protected peptide, BocTyr-Gly-Gly-Phe-Leu-Arg(Pmc)NH_2, prepared from the Rink resin and the MBHA resin were characterized by HPLC, FAB-MS (see Table 9.1), and amino acid analysis (see Table 10.2).

The HPLC analysis for both protected peptides were carried out in the same manner. Twenty μL of the crude protected peptide after cleavage (approximately 1mg/mL in HPLC MeOH) was injected onto the column and eluted using an isocratic system of 40% H_2O and 60% MeOH.

FAB-MS and amino acid analysis were performed as described in Chapter 4, except resin samples were hydrolyzed with HCl plus propionic acid.

Table 9.1 HPLC[1] and FAB-MS Data

Analysis of Model Compound

BocTyr-Gly-Gly-Phe-Leu-Arg(Pmc)NH_2

Resin	%B	Isocratic HPLC R_v (min)	% Purity	FAB-MS (M+1)
Rink	60	12.94	98.0	1077
MBHA	60	13.46	96.6	1077

[1] Zorbax Protein Plus Column, 0.46 × 25 cm;
Solvent A = HPLC H_20
Solvent B = HPLC MeOH

CHAPTER 10

RESULTS AND DISCUSSION

10.1 Protected Peptide Amides

The path to the preparation of a protected peptide amide began by first considering the commercially available resins. The PAL resin came out of the collaborative effort between Milligen/Biosearch and George Barany (Albericio and Barany, 1987). This resin produces a peptide amide, but the peptide is cleaved from the resin using 95% TFA which also removes the side chain protecting groups. Thus, it was not a viable option for the preparation of a protected peptide amide.

The Pepsyn KB resin is a polymerized gel within a rigid Kieselguhr matrix (Arshady et al., 1981). When combined with the 4-hydroxymethylbenzoic acid linker (Linker B), a peptide amide may be produced upon cleavage by methanolic ammonia. However, this resin was designed to be used with a continuous-flow system and not a batch synthesizer like the Biosearch 9500, and was not considered. Since the B Linker can be attached to a gel resin and cleaved as described with the Kieselguhr composite resin, it seemed a likely candidate for use. The gel resin was described to be used with a batch-type instrument.

During the course of this project two polystyrene "Rink" resins became commercially available. One resin produced a protected peptide acid upon weak TFA cleavage while the second produced a peptide amide (Rink, 1987). Another polystyrene resin was found in the literature which was a 9-xanthenyl resin (Sieber, 1987). Since this resin, which can be cleaved using a TFA/1,2-dichloroethane 2:98 (v/v) solution pumped through a glass column (Sieber, 1987), was not commercially available, the amide producing Rink resin was chosen over it.

The failure of both the gel and Rink resin to adequately meet the needs of producing a protected peptide amide led to the development and synthesis of a second polystyrene-based resin. The attachment of Linker B to an MBHA resin to produce a protected peptide amide upon ammonia cleavage was attempted.

10.2 Gel Resin

10.2.1 Functionalization

The gel resin necessitated functionalization of the sarcosine methyl ester before a synthesis could be performed on the resin. The gel resin was prepared for synthesis according to a modification of MilliGen Technical Note 4.20. (see also Arshady et al., 1981). The functionalization process modified the sarcosine methyl ester groups on the resin to primary amine sites by

reacting the ester groups with ethylenediamine. Ala was incorporated as an internal standard instead of the Nle that MilliGen suggested because a potential model peptide included Nle. FmocAla was incorporated as an internal standard using the OPfp active ester and the Fmoc group removed. The incorporation of the internal standard allowed quantification of the resin substitution by amino acid analysis. As the final step in the functionalization process the 4-hydroxymethylbenzoic acid linker (B Linker) was coupled to the amine group of the internal standard. The B Linker was used in order to produce the peptide amide upon cleavage with ammonia (see Figure 10.1).

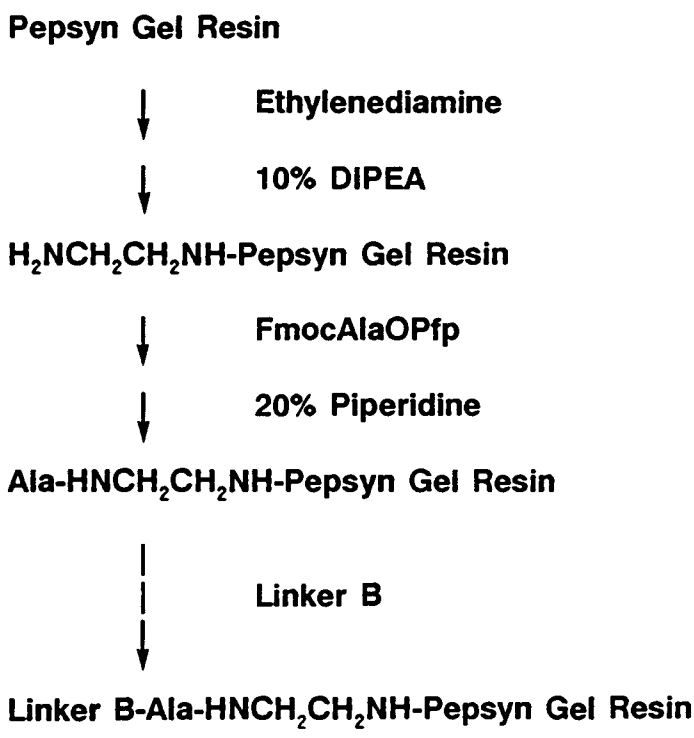


Figure 10.1 Functionalization of Pepsyn Gel Resin

10.2.2 Esterification

Once the resin was functionalized, the C-terminal FmocArg(Mtr) was coupled to the hydroxyl group of the linker to form an ester. The coupling protocol used for esterification was the symmetrical anhydride method. This method used dicyclohexylcarbodiimide (DCC) to prepare the symmetrical anhydride. Symmetrical anhydrides are very reactive species and must be prepared immediately prior to use. The FmocArg(Mtr) symmetrical anhydride was coupled to the functionalized resin using DMAP as a catalyst (see Figure 10.2). The DMAP was used only in a catalytic amount to minimize the potential for racemization.

During the final washes of the esterification reaction the resin became a solidified gelatinous mass that completely retained the mold of the reaction vessel when removed.

Reactions

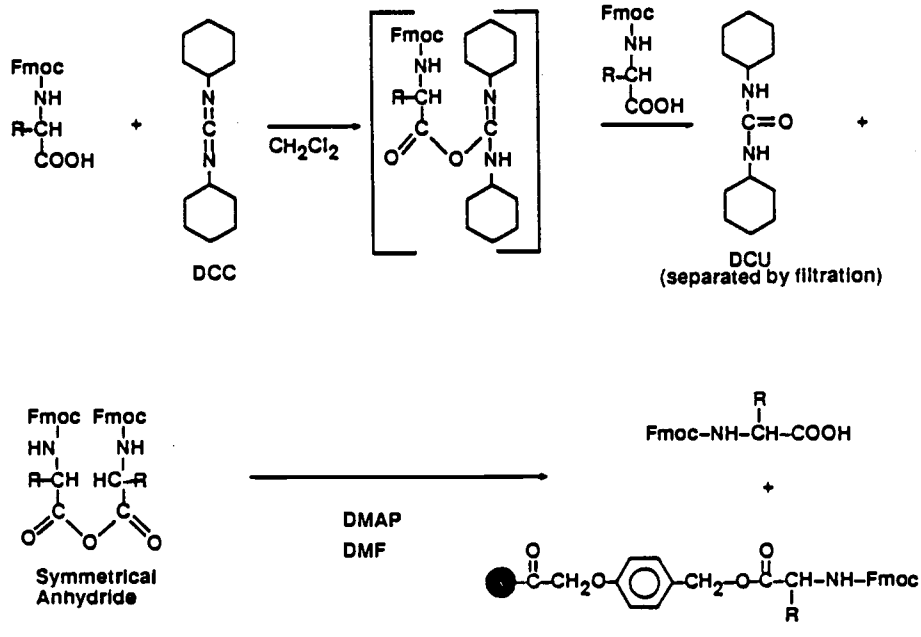


Figure 10.2 Esterification of Resin

Source: MilliGen Technical Note 4.30

10.2.3 Synthesis of Model

The synthesis of a model peptide, BocTyr-Gly-Gly-Phe-Leu-Arg(Mtr)NH_2, was attempted using the functionalized resin, but the synthesis was unsuccessful. Due to the viscosity of the gel resin, the normal automation of the peptide synthesizer was halted at each synthesis step (approximately 1000 steps) due to the inability of the reaction vessel to empty. At the end of the synthesis the recovery of peptide-resin was 28% of the expected weight. Therefore it was concluded that this was not a viable resin for the needs of this project and was abandoned.

The gel resin swells up to twenty times its dry volume in DMF (MilliGen Technical Note 4.20.). The swelling, while enhancing contact between reagents and the resin, makes the resin extremely fragile. It is speculated that resin decomposition caused fines to be generated which lead to the failure of a instrument valve only a short time after the synthesis of the gel resin.

Although the recovery of peptide synthesized was poor, cleavage of the peptide from the resin was attempted. The overall recovery of the peptide from the resin was only 16.9%, but relative to the expected amount of peptide for this quantity of resin the recovery was 60.3%. This indicates that the main loss was during the synthesis, not during the cleavage. Thus this approach might be applicable under altered synthesis conditions. The synthesis of the

protected peptides might be successful using the Kieselguhr composite (Pepsyn KB) resin on a continuous-flow instrument.

10.2.4 Quantitative Loading of Resin

In order to determine the quantitative loading of the resin, the resin was subject to both quantitative Fmoc determination and amino acid analysis. The Fmoc group is an excellent chromophore for UV-absorbance. In this method, the Fmoc group is removed quantitatively from the resin and the quantity is measured spectrophotometrically (see Figure 10.3).

The amino acid analysis showed a loading of 0.25 mmole/g (102.0% expected) at Ala and 0.11 mmole/g (47.8%) at Arg. The data from the quantitative Fmoc analysis showed a substitution of 0.16 mmole/g (65.5%) at Arg. These data suggest that the loading of Ala to the ethylenediamine spacer went to completion, but that the loading of the Arg to the linker was only about 50% complete. This was checked by directly comparing Ala internal standard to Arg from the same sample. The Arg substitution was 45.9% of the Ala substitution.

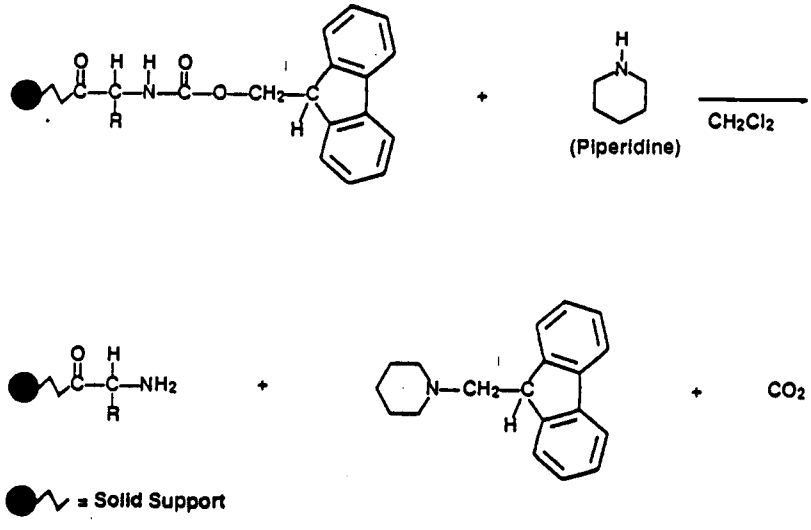


Figure 10.3 Quantitative Fmoc Determination

Source: MilliGen Technical Note 3.10

10.3 Rink Resin

10.3.1 Synthesis

In contrast to the polyacrylamide gel resin, the "Rink" polystyrene resin provided an excellent medium for automated peptide synthesis on a batch-type instrument. The model peptide, BocTyr-Gly-Gly-Phe-Leu-Arg(Pmc)NH_2, was synthesized on the resin without the need for any resin functionalization. The Arg protecting group was changed to Pmc since it is more readily removed than Mtr (Riniker and Hartmann, 1990). The coupling additive HOBt was not used due to the acid lability of the resin (Rink, 1987). The synthesis was extremely successful with essentially the theoretical expected weight gain (99.9%).

10.3.2 Cleavage

The problem with the "Rink" resin was in the cleavage process. The resin was cleaved with 2% TFA in DCM which only resulted in a peptide recovery of 10% even after doubling the reaction time. Since the peptide was not removed from the resin even after doubling the reaction time a stronger acid concentration was examined. Althouth the stronger acid would remove all the side chain protecting groups it was used in an attempt to cleave the peptide from the resin. The harsher cleavage condition (modified Reagent R), only increased the peptide recovery to 27%.

10.3.3 Quantitative Resin Loading

A modified model peptide, Fmoc(tBu)-Gly-Gly-Phe-Leu-Arg(Pmc)-Rink Resin, was synthesized to replace BocTyr with FmocTyr(tBu) such that the peptide could be subjected to quantitative Fmoc analysis. This quantitative Fmoc analysis could be compared to amino acid analysis data.

The observed substitution by quantitative Fmoc analysis matched quite closely with the expected substitution. The final loading was 0.33 mmol/g by quantitative Fmoc (91.7% expected), but was only 0.24 mmole/g (67.8% expected) by amino acid analysis (see Table 10.1). The difference in loading between methods could be explained by weighing errors or incomplete hydrolysis of the sample for amino acid analysis.

Amino acid analysis data confirmed that cleavage of the peptide from the resin was the problem, with 65.3% of the model peptide remaining on the resin.

Table 10.1 Results of Substitution Analysis on Rink Resin

Comparison of Rink Resin Substitution
Using Quantitative Fmoc

	Expected Substitution	Observed Substitution	% Expected
FmocArg(Pmc)-Rink Resin	0.46	0.44	95.7
FmocLeu-Arg(Pmc)-Rink Resin	0.44	0.39	88.6
FmocPhe-Leu-Arg(Pmc)-Rink Resin	0.41	0.37	90.2
FmocGly-Phe-Leu-Arg(Pmc)-Rink Resin	0.40	0.38	95.0
FmocGly-Gly-Phe-Leu-Arg(Pmc)-Rink Resin	0.39	0.34	87.2
FmocTyr(tBu)-Gly-Gly-Phe-Leu-Arg(Pmc)-Rink Resin	0.36	0.33	91.7

10.3.4 Analysis of Peptide

The peptide recovered was subjected to amino acid analysis, FAB-MS and HPLC. HPLC data indicated the product was relatively pure (see Table 9.1). FAB-MS confirmed the molecular weight of 1077 expected for the protected peptide amide. The data therefore confirmed that the peptide recovered after cleavage was the desired product.

The results of the amino acid analysis of the resin indicated that the synthesis was effective (see Table 10.2). The expected amino acid substitution indicated that 79.5% of the peptide was still attached to the resin. Even after cleavage with modified Reagent R (87% TFA, 5% thioanisole, 3% ethanedithiol, 5% water) the peptide-resin bond was not broken. The reasons for the poor recovery of the peptide, even after treatment with concentrated TFA, aren't clear at this time. Possible explanations will be investigated in our laboratory.

Even though the use of the "Rink" resin was not optimal, a precursor peptide (BocTyr(tBu)-Gly-Gly-Phe(NO_2)-Leu-Arg(Pmc)-Arg(Pmc)-Ile-Arg(Pmc)-DPro-Lys(Boc)NH_2) was synthesized on it in an attempt to reach the ultimate goal of preparing an affinity labeled peptide. The cleavage of this precursor from the resin using the weak acid conditions resulted in only a 1% yield and was abandoned at that stage. Again, amino acid analysis confirmed that 89.5% of the peptide was still attached to the resin (see Table 10.2).

Table 10.2 Amino Acid Analysis of Model Compound

BocTyr-Gly-Gly-Phe-Leu-Arg(Pmc)NH_2

	Tyr(1)	Gly(2)	Phe(1)	Leu(1)	Arg(1)	Substitution (mmol/g)	%Expected Substitution
Rink Model[1]	0.877	2.095	1.029	1.054	0.944	0.322	82.1
Rink Model[2]	0.844	2.104	1.022	1.047	0.984	0.256	
MBHA Model[1]	0.820	2.025	1.054	1.015	1.087	0.331	105.7
MBHA Model[2]	0.843	2.101	1.020	1.009	1.027	0.151	

	Tyr(1)	Gly(1)	Phe(1)*	Leu(1)	Arg(3)	Ile(1)	Pro(1)	Lys(2)	Substitution (mmol/g)	%Expected
Rink Precursor[1]	0.618	1.809	1.139	1.002	3.018	0.953	1.015	1.064	0.199	80.0
Rink Precursor[2]	0.640	1.790	1.207	1.002	2.992	0.939	1.014	1.057	0.222	

	Tyr(1)	Gly(2)	Phe(1)	Leu(1)	Arg(1)	% Peptide
Peptide from MBHA	0.989	1.955	1.025	1.020	1.011	92.3

Model: BocTyr-Gly-Gly-Phe-Leu-Arg(Pmc)NH_2
Precursor: BocTyr(tBu)-Gly-Gly-Phe-Leu-Arg(Pmc)-Arg(Pmc)-Ile-Arg(Pmc)-DPro-Lys(Boc)NH_2

[1] resin before cleavage
[2] resin after cleavage
* Phe(p-NO_2)

10.4 MBHA Resin

10.4.1 Functionalization

The MBHA resin was functionalized using a protocol modified from the gel resin functionalization. Since the MBHA resin was designed for use with Boc synthesis a Boc chemical protocol was used for this part of the synthesis. Ala was incorporated as the internal standard for the resin using BocAla and DIPCDI. A capping step was incorporated after coupling the BocAla to the resin to insure any unreacted sites were acylated (see Chapter 4 for a discussion of the capping procedure). 4-Hydroxymethylbenzoic acid (Linker B, see Section 10.2) was incorporated once again as the OPfp active ester (see Figure 10.4).

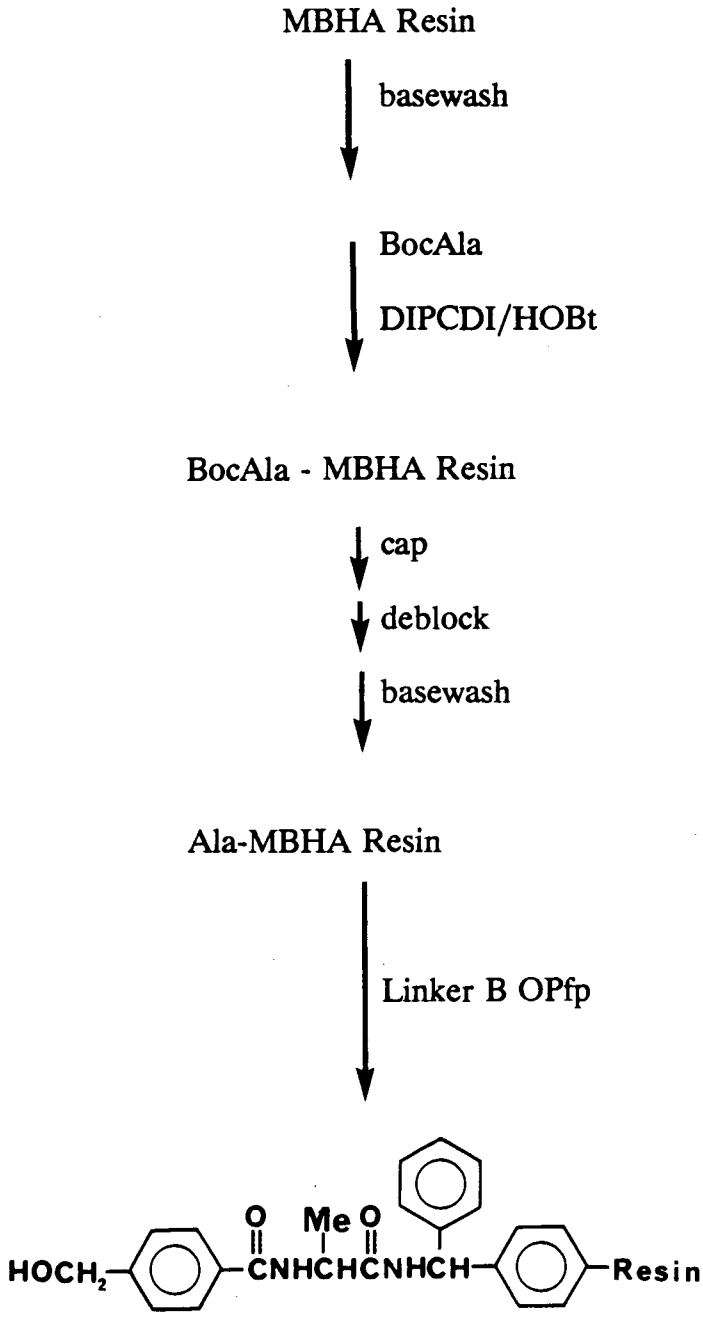


Figure 10.4 Functionalization of MBHA Resin

10.4.2 Esterification

The MBHA resin was esterified analogously to the gel resin except FmocArg(Pmc) was used in place of the FmocArg(Mtr) (see Figure 10.5). The chemistry was changed at this point from Boc to Fmoc. Both chemical protocols were used to take advantage of the optimized chemistry for the MBHA resin. The Fmoc chemistry was integrated at the esterification stage in preparation for synthesis of the model peptide. The differences in the protocol between the gel and MBHA resins were that the reaction time was substantially increased, and the MBHA resin was acylated after coupling of the FmocArg(Pmc) to the resin. The reaction time for the coupling of the symmetrical anhydride to the resin was lengthened because the esterification reaction with the gel resin appeared incomplete. The MBHA resin was capped to insure all unreacted sites were acylated.

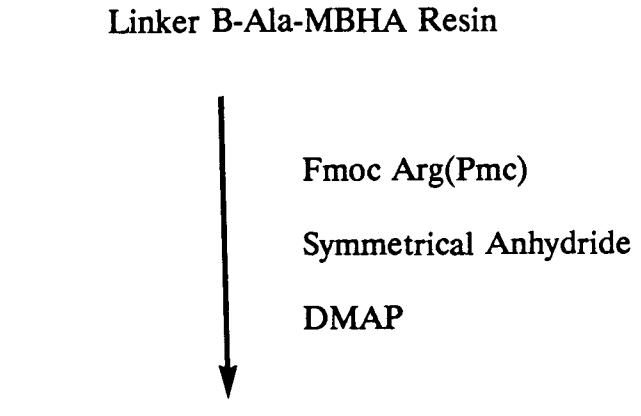


Figure 10.5 Esterification of MBHA Resin

10.4.3 Synthesis of Model

The model peptide, BocTyr-Gly-Gly-Phe-Leu-Arg(Pmc)NH_2 was synthesized in the same manner as the model compound on the "Rink" resin, except that the C-terminal FmocArg(Pmc) was already attached to the resin. The chemical protocol used in the synthesis of the model peptide utilized the Fmoc protocol as opposed to the Boc protocol used for synthesis on the unfunctionalized resin in part one of this thesis. The synthesis of the model peptide on the resin was successful with a 88% expected weight gain and data from the amino acid analysis indicating that the relative amounts of each amino acid were as expected (see Table 10.2).

10.4.4 Cleavage of Model

The peptide was cleaved from the resin using the nucleophilic attack of the NH_3 in iPrOH. The cleavage was also successful with a recovery of 81% protected peptide. Isopropanol was used to avoid the potential for methyl ester formation. Initially the resin was cleaved for 4 hour, but lack or recovery and amino acid analysis data suggested that the peptide was still on the resin; since the concentration of Arg on the resin after cleavage matched (99.8%) the concentration of the Ala internal standard. In an attempt to improve peptide recovery after iPrOH/NH_3 cleavage, the reaction time was lengthened to 4 days for a increased yield of 52.7%. The inclusion of a DMF

as a cosolvent was also attempted with the 4 day cleavage but failed to improve recovery after cleavage.

As with the synthesis of the model compound, amino acid analysis data indicated that the relative amounts of each amino acid were as expected (see Table 10.2).

10.4.5 Quantitative Loading of Resin

The protected peptide from the MBHA resin was subjected to the same quantitative loading analysis as discussed in section 10.2.4. The substitution results by amino acid analysis showed a substitution larger than 100% which indicated that the original substitution of the MBHA resin was greater than the stated value. The Arg substitution was 92.3% of the Ala substitution which was much better than for the gel resin (46%) indicating a more effective esterification. This was probably due to the increased coupling time from 20 h for the MBHA resin vs 2 hr for the gel resin.

The substitution at the C-terminal Arg(Pmc) was determined to be 0.39 (139.9%) by amino acid analysis and 0.39 (107.8%) by quantitative Fmoc. Amino acid analysis data showed that cleavage was successful with only 19.2% of the peptide remaining on the resin.

10.4.6 Analysis of Peptide

The protected peptide recovered was subjected to the same characterization as was described for the model peptide from the "Rink" resin (see section 10.3.3). HPLC data indicated that the relative purity of the product was high (see Table 9.1). FAB-MS confirmed the molecular weight of 1077 expected for the protected peptide amide and amino acid analysis confirmed the amino acid composition of the peptide (see Table 10.2). As with the "Rink" resin, these data indicated that the peptide recovered after cleavage was the desired product.

CHAPTER 11

CONCLUSIONS

Three different resins were analyzed in an effort to prepare a protected peptide amide using the Fmoc chemical protocol. The results of that effort indicated that the synthesis of a protected peptide amide was not a trivial endeavor.

The polyamide "gel" resin was found not to be compatible with a batch-type instrument. The resin transformed during esterification from a soft gel bead into a solid gelatinous mass that resulted in a very poor expected recovery of resin after the synthesis (28%). This viscous resin completely negated the benefits of the automated instrument by requiring manual filtration of the resin at each step of the synthesis. Further, the decomposition of the resin during synthesis resulted in the loss of function of an instrument valve shortly after the synthesis. Thus, the polyamide gel resin was not a feasible alternative for the production of a protected peptide on our instrument.

On the other hand, the "Rink" polystyrene resin provided an excellent medium for automated peptide synthesis. The flaw with this resin was its inability to release the protected peptide after synthesis. The cleavage of the

peptide from the resin was not feasible using either weak TFA cleavage (10% recovery) or even concentrated TFA cleavage conditions (29% recovery).

Even though the use of the "Rink" resin was not optimum, a precursor peptide was synthesized on it in an attempt to reach the ultimate goal of preparing an affinity labeled peptide. The cleavage of this precursor from the resin using the weak acid conditions resulted in only a 1% yield and was abandoned at that stage.

Amino acid analysis data on both the model and precursor peptide indicated that the synthesis was effective and that the peptide was present on the resin in its entirety, but that the cleavage reactions did not break the peptide-resin bond. Results from this project indicate that the "Rink" resin does not appear to be a feasible support for either the production of a peptide amide or a protected peptide amide fragment.

The modified polystyrene MBHA resin designed and synthesized during this project prevailed as the only feasible resin of the three investigated, to adequately produce a protected peptide fragment. The synthesis was compatible with the batch instrument (71.3% expected weight gain) and the protected peptide could be cleaved from the resin (52.7% recovery). Further research on this type of resin may include improving the synthesis efficiency on the resin and optimizing cleavage conditions for a higher recovery after synthesis. The MBHA resin prepared for this project could have wide ranging flexibility for use in the preparation of protected

peptide amides once the conditions for resin preparation and cleavage of the peptide from the resin are optimized.

Protected peptide fragments are useful precursors since they can be used as intermediate species which can be modified further to produce novel peptides. The solid supports for the production of these fragments seem to be the stumbling block for their preparation.

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APPENDIX

APPENDIX

Table A.1 Structure-Activity Relationships of Dynorphin Analogues

	Opioid Activity[a]		Receptor Binding[b]			
Analogue	IC_{50} (nM)	Relative Potency	IC_{50} (nM)	Relative Potency	(μ:δ:κ)	Reference
Dynorphin A-(1-17)[1]	0.28±0.04	100	0.23±0.02	100	7:27:1	Gairin et al, 1986
Dynorphin A-(1-13)[2]	0.7±0.1	100	2.7±0.3	100		Turcotte et al, 1984
Dynorphin A-(1-13)[3]	0.66±0.01	100	0.11±0.01	100	4:40:1	Lemarie et al., 1986
Dynorphin A-(1-13)[4]	0.33	100				Chavkin and Goldstein, 1981
Dynorphin A-(1-13)[5]	0.7±0.1	100	2.1±0.4	100		Lemaire and Turcotte, 1986
Dynorphin A-(1-13)[6]	0.163±0.027	100				Schiller et al., 1982
Dynorphin A-(1-11)[7]	2.88±0.28	100	0.128±0.038	100	21:83:1	Gairin et al, 1986
Dynorphin A-(1-10)NH_2[8]	30	100				Rezvani et al., 1984
Dynorphin A-(1-13)[9]	1.46±0.41	100				Schiller et al., 1988
Dynorphin A-(1-13)[10]			0.045	100	5:10:1	Nakajima et al., 1988
Dynorphin A-(1-9)[11]			0.29±0.03	100	11:11:1	Paterson et al., 1984

[a] Guinea pig ileum

[b] Guinea pig cerebellum

Table A.1 Continued

Structure-Activity Relationships of Dynorphin Analogues

	Opioid Activity[a]		Receptor Binding[b]			
Analogue	**IC_{50} (nM)**	**Relative Potency**	**IC_{50} (nM)**	**Relative Potency**	**(μ:δ:κ)**	**Reference**
Tyr^1 Substitution						
[Ser^{14}]Dyn A-(2-17)[4]	inactive at 10 μM					Chavkin and Goldstein, 1981
α-N-Me Dyn A-(1-13)NH_2[4]	0.42	79				Chavkin and Goldstein, 1981
[Ala^1] Dyn A-(1-13)[2]	750±30	<0.1	1400±210	0.2		Turcotte et al., 1984
Gly^2 Substitution						
[Ala^2] Dyn A-(1-13)[2]	104±32	0.6	13.5±0.4	20		Turcotte et al., 1984
[Ala^2] Dyn A-(1-13)[5]	104±32	0.6	2.9±0.6	72		Lemaire and Turcotte, 1986
[D-Ala^2] Dyn A-(1-13)NH_2[4]	1.94	17				Chavkin and Goldstein, 1981
[D-Ala^2] Dyn A-(1-11)[4]	2.91	11				Chavkin and Goldstein, 1981
[D-Ala^2] Dyn A-(1-10)[4]	14.4	2				Chavkin and Goldstein, 1981
[D-Ala^2] Dyn A-(1-9)[11]			2.26±0.34	13		Paterson et al., 1984
Gly^3 Substitution						
[Ala^3] Dyn A-(1-13)[2]	2.0±0.3	35	21.5±7	12		Turcotte et al., 1984

Table A.1 Continued

Structure-Activity Relationships of Dynorphin Analogues

	Opioid Activity[a]		Receptor Binding[b]			
Analogue	**IC_{50} (nM)**	**Relative Potency**	**IC_{50} (nM)**	**Relative Potency**	**(μ:δ:κ)**	**Reference**
Phe^4 Substitution						
[Ala^4] Dyn A-(1-13)[2]	700±20	0.1	750±35	0.4		Turcotte et al., 1984
[$Phe(NO_2)^4$]Dyn A-(1-13)[6]	0.705±0.259	23				Schiller et al, 1982
[Trp^4]Dyn A-(1-13)[6]	1.11±0.04	15				Schiller et al, 1982
[Trp^4]Dyn A-(1-13)[5]	88±2	0.8	3.1±0.1	68		Lemaire and Turcotte, 1986
Leu^5 Substitution						
[Ala^5]Dyn A-(1-13)[2]	14±4	5	45.0±1.1	6		Turcotte et al., 1984
[Met^5]Dyn A-(1-10)NH_2[8]	30	100				Rezvani et al., 1984
Arg^6 Substitution						
[D-Arg^6]Dyn A-(1-13)			50-70			Wuster et al., 1980
[Ala^6]Dyn A-(1-13)[2]	23±6	3	19.2±0.8	14		Turcotte et al., 1984
Arg^7 Substitution						
[Ala^7]Dyn A-(1-13)[2]	19±5	4	10.0±1.2	27		Turcotte et al., 1984

Table A.1 Continued

Structure-Activity Relationships of Dynorphin Analogues

	Opioid Activity[a]		Receptor Binding[b]			
Analogue	**IC_{50} (nM)**	**Relative Potency**	**IC_{50} (nM)**	**Relative Potency**	**(μ:δ:κ)**	**Reference**
Ile^8 Substitution						
[Ala^8]Dyn A-(1-13)[2]	1.4 ± 0.1	50	0.3 ± 0.0	900		Turcotte et al., 1984
[Ala^8]Dyn A-(1-13)[3]	0.96 ± 0.12	69	0.05 ± 0.01	220	9:148:1	Lemaire et al., 1986
[D-Ala^8]Dyn A-(1-13)[3]	1.89 ± 0.25	35	0.08 ± 0.01	137	6:97:1	Lemaire et al., 1986
[Trp^8]Dyn A-(1-13)[3]	1.58 ± 0.27	42	0.10 ± 0.01	110	12:46:1	Lemaire et al., 1986
[D-Trp^8]Dyn A-(1-13)[3]	1.72 ± 0.34	38	0.22 ± 0.06	50	8:30:1	Lemaire et al., 1986
[Met^8]Dyn A-(1-10)NH_2[8]	50	60				Rezvani et al., 1984
[Nle^8]Dyn A-(1-10)NH_2[8]	400	8				Rezvani et al., 1984
Arg^9 Substitution						
[Ala^9]Dyn A-(1-13)[2]	5.5 ± 0.8	13	7.4 ± 0.8	36		Turcotte et al., 1984

Table A.1 Continued

Structure-Activity Relationships of Dynorphin Analogues

	Opioid Activity[a]		Receptor Binding[b]			
Analogue	IC_{50} (nM)	Relative Potency	IC_{50} (nM)	Relative Potency	(μ:δ:κ)	Reference
Pro^{10} Substitution						
[Ala^{10}]Dyn A-(1-13)[2]	3.3±0.7	21	3.3±0.7	21		Turcotte et al., 1984
[Trp^{10}]Dyn A-(1-13)[3]	0.98±0.13	67	0.41±0.13	27	4:18:1	Lemaire et al., 1986
[D-Trp^{10}]Dyn A-(1-13)[3]	2.15±0.49	31	0.44±0.01	25	6:24:1	Lemaire et al., 1986
[D-Pro^{10}]Dyn A-(1-13)[3]	1.10±0.45	60	0.09±0.01	122	14:64:1	Lemaire et al., 1986
[D-Pro^{10}]Dyn A-(1-11)[7]	3.32±0.23	87	0.032±0.008	400	63:233:1	Gairin et al., 1986
[D-Pro^{10}]Dyn A-(1-11)			13±4			Gairin et al., 1988
[Thz^{10}]Dyn A-(1-10)NH_2[8]	60	50				Rezvani et al., 1984
Lys^{11} Substitution						
[Ala^{11}]Dyn A-(1-13)[2]	7.6±0.4	9	8.7±0.9	31		Turcotte et al., 1984

Table A.1 Continued

Structure-Activity Relationships of Dynorphin Analogues

	Opioid Activity[a]		Receptor Binding[b]			
Analogue	**IC_{50} (nM)**	**Relative Potency**	**IC_{50} (nM)**	**Relative Potency**	**(μ:δ:κ)**	**Reference**
Multiple Substitutions						
[D-Ala^2,D-Arg^6] Dyn A-(1-13)	3-5					Wuster et al., 1980
[D-Ala^2,D-Arg^{6,7,9}] Dyn A-(1-9)^{11}			7.25 ± 1.79		0.97:0.06:1	Paterson et al, 1984
[D-Ala^2,D-Arg^{6,7,9}] Dyn A-(1-9)NH_2^{11}			2.72 ± 0.64		0.14:0.21:1	Paterson et al, 1984
[D-Arg^{6,7,9}] Dyn A-(1-9)^{11}			1.42 ± 0.40		0.51:1.35:1	Paterson et al, 1984
[D-Arg^{6,7,9}] Dyn A-(1-9)NH_2^{11}			0.50 ± 0.15		0.78:4.96:1	Paterson et al, 1984
[D-Trp^8,D-Pro^{10}]Dyn A-(1-11)^7	2.84 ± 0.26	101	0.049 ± 0.003	261	12:111:1	Gairin et al., 1986
[Phe^{8,12},Lys^{10}] Dyn A-(1-13)^{10}			0.035	129	5:6:1	Nakajima et al., 1988
[Phe^{8,12},Pro^{10}] Dyn A-(1-13)^{10}			0.035	129	6:10:1	Nakajima et al., 1988

Table A.1 Continued

Structure-Activity Relationships of Dynorphin Analogues

	Opioid Activity[a]		Receptor Binding[b]			
Analogue	IC_{50} (nM)	Relative Potency	IC_{50} (nM)	Relative Potency	(μ:δ:κ)	Reference
Cyclic Compounds						
[D-Cys^2, Cys^5] Dyn A-(1-13)^6	0.0317 ± 0.0058	514				Schiller et al., 1982
[Orn^5,Asp^8] Dyn A-(1-13)NH_2^{9}	1970 ± 490	7				Schiller et al., 1988
[Orn^5,Asp^{10}] Dyn A-(1-13)NH_2^{9}	667 ± 63	0.22				Schiller et al., 1988
[Orn^5,Asp^{13}] Dyn A-(1-13)NH_2^{9}	687 ± 73	0.21				Schiller et al., 1988

Table A.1 Continued

Structure-Activity Relationships of Dynorphin Analogues

Analogue	Opioid Activity[a] IC_{50} (nM)	Relative Potency	Receptor Binding[b] IC_{50} (nM)	Relative Potency	(μ:δ:κ)	Reference
Antagonists						
[Ala^2,Trp^4] Dyn A-(1-13)^5	5500 ± 200	<0.1	incactive			Lemaire and Turcotte, 1986
[D-Trp^{2,8},D-Pro^{10}]Dyn A-(1-11)^7	8890 ± 660	0.03	153 ± 15	0.08	0.7:4:1	Gairin et al, 1986
[D-Trp^{3,8},D-Pro^{10}]Dyn A-(1-11)^7	222 ± 8	1.3	19.6 ± 1.7	0.7	17:53:1	Gairin et al., 1986
[D-Trp^{4,8},D-Pro^{10}]Dyn A-(1-11)^7	> 10,000	<0.03	73 ± 25	0.2	2:9:1	Gairin et al., 1986
[D-Trp^{5,8},D-Pro^{10}]Dyn A-(1-11)^7	69 ± 7	4.2	20.6 ± 1.4	0.6	1:15:1	Gairin et al., 1986
[D-Trp^{2,4,8},D-Pro^{10}]Dyn A-(1-11)^7	> 10,000	<0.03	23.8 ± 3.2	0.5	2:6:1	Gairin et al., 1986
N,N-diallyl-D-Pro^{10}]Dyn A-(1-11)			> 10,000			Gairin et al., 1988
N,N-diallyl-[Aib^{2,3}]-D-Pro^{10}]Dyn A-(1-11)			> 10,000			Gairin et al., 1988

[1] Compared to Dynorphin A-(1-17)[1]
[2] Compared to Dynorphin A-(1-13)[2]
[3] Compared to Dynorphin A-(1-13)[3]
[4] Compared to Dynorphin A-(1-13)[4]
[5] Compared to Dynorphin A-(1-13)[5]
[6] Compared to Dynorphin A-(1-13)[6]
[7] Compared to Dynorphin A-(1-11)[7]
[8] Compared to Dynorphin A-(1-10)NH_2[8]
[9] Compared to Dynorphin A-(1-13)[9]
[10] Compared to Dynorphin A-(1-13)[10]
[11] Compared to Dynorphin A-(1-9)[11]