An Abstract of the Thesis of

Kristin Renee Snyder for the degree of Master of Science in Biochemistry and Biophysics presented on May 20, 1993.

Title: Synthesis and Opioid Activity of Dynorphin A Analogues

Abstract approved: _____

Jane V. Aldrich

Throughout history the opioid ligand morphine has been used as one of the most efficacious analgesics. However, because of its high abuse potential and undesirable side effects, there is an ongoing search for an opioid replacement without these characteristics. A prerequisite to developing new drugs is an understanding of the receptor-ligand interactions which involve both the structure and charge requirements of the receptor binding site and the ligand. Described here are studies which examine both the charge requirements of the C-terminal "address" portion of dynorphin A (Dyn A), (Chap. 1), and the structure requirements of the N-terminal "message" sequence of this endogenous κ-opioid ligand (Chap II).

The study in Chapter I involved removing the positive charge at each of residues 6, 7, 9, 11, and 13 in Dyn A-(1-13)NH_2 by replacing each amino acid in these positions by N^{ε}-acetyllysine (Lys(Ac)). The results substantiated earlier studies that Arg^7, Lys^{11}, and Lys^{13} are important for κ-opioid potency and selectivity. It was found that a positive charge at position 6 is required for high κ-opioid selectivity, but potency is not affected by its elimination. Of the five basic residues of Dyn A, only the charge of Arg^9 can be removed with no effect upon κ-opioid potency or selectivity.

No conformational structure for Dyn A can be agreed upon based on the results of biophysical experiments. Incorporation of conformational constraints into the peptide and examination of structure-activity relationships is an alternative method for examining the bioactive conformation of peptides. Chapter II describes the synthesis and pharmacological evaluation of Dyn A-(1-13)NH_2 analogues that have been substituted with *cis*- or *trans*-4-aminocyclohexanecarboxylic acid (ACCA) for Gly^2-Gly^3. Although both analogues had low affinity for opioid receptors, they are the first reported κ-selective Dyn A analogues constrained in the "message" sequence.

Synthesis and Opioid Activity of Dynorphin A Analogues

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Professor of Pharmacy, Appointed by Biochemistry and Biophysics, in charge of major

Redacted for Privacy

Head of Department of Biochemistry and Biophysics

Redacted for Privacy

Dean of Graduate School

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Typed by Kristin Renee Snyder

Acknowledgement and Contribution of Authors

I would like to give due credit to the co-authors of the papers that comprise chapters I and II of this thesis. Sandra Story synthesized and purified [Lys]^{9}- and [Lys(Ac)]^{9}Dyn A-(1-13)NH_2, and helped develop some of the synthesis and purification methods used. Mia Heidt synthesized and purified [Lys]^{6}- and [Lys(Ac)]^{6}Dyn A-(1-13)NH_2, and helped develop some of the cleavage methods used. Dr. Thomas Murray and his technicians Tom Jacobsen and Valerie Caldwell developed and performed all binding assays. Dr. Gary DeLander and his technician Marty Knittle developed and performed all smooth muscle assays. Thanks to all.

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Synthesis and Opioid Activity of Dynorphin A Analogues

Introduction

History of Opioids[1,2]

The ancient Sumerians (4000 B.C.) and Egyptians (2000 B.C.) may have known the psychological effects of opium and its usefulness in controlling pain and diarrhea, but the first undisputed reference to this juice of the poppy *Papaver sominiferum* is from the Greeks in 3 B.C. Over the years opium was used for medicinal purposes in Arabia, the Orient, Europe, and to some extent the United States. However, with unrestricted availability came abuse and addiction to opium and its main active ingredient morphine. While morphine is still one of the most effective and widely used pain-relieving drugs, its undesirable side effects (respiratory and pulmonary depression, constipation, and abuse potential) have stimulated a large research effort to develop non-addictive analgesics. Although this has not been achieved, many clinically useful drugs have been produced. More importantly, this research has resulted in the discovery of multiple opioid receptors and endogenous opioid peptides in vertebrate animals including humans. With more knowledge of the structure, conformation, and biochemistry of these peptides and receptors perhaps someday a non-addictive opioid analgesic without these side effects will be developed.

Opioid Receptors and Opioid Action

Multiple Opioid Receptor Types

The first proposal of a receptor for opiates was by Beckett and Casy, based on activities of stereoisomers.[3] Many years later Martin's lab proposed the existence of three different receptor types, mu (μ), kappa (κ), and sigma (σ), to explain

the different behavioral and physical effects of morphine, ketazocine, and N-allyl-normetazocine, SKF 10,047, in chronic spinal dogs.[4,5] The effects seen with morphine administration included analgesia, miosis, bradycardia, hypothermia, and indifference to environmental stimuli. Ketazocine caused miosis, general sedation, and depression of flexor reflexes, while SKF 10,047 caused mydriasis, increased respiration, tachycardia, and delirium. Since then, the σ receptor has been designated non-opioid because the behavioral effects induced by its ligands (benzomorphans) are not blocked by naloxone, a high affinity antagonist often used to define compounds as opioids.[6,7] A true third opioid receptor is the delta (δ) receptor type, which is antagonized by naloxone and distinctly differs from μ or κ receptors.[8,9]

Although antagonism by naloxone has been traditionally used to characterize opioid receptors, there are other ligands now being used to characterize the different receptor types (Table 1).[10,11]

Location of Receptors

The locations of μ, δ, and κ opioid receptors have been studied in tissues of several species using autoradiographic techniques. A tritiated general opioid ligand is added to the tissue slices, then a ligand specific for one receptor type is added and its ability to displace the general ligand is measured for quantitative results. Opioid receptors have been found to be located in physiologically relevant areas of both the central nervous system (CNS) and peripheral tissues. In rat and guinea pig, μ receptors are found in many parts of the CNS including the pyriform cortex,[12] layers III-VI of the cerebral cortex, N. accumbens, amygdala, olfactory bulb, striatal patches and streaks,[13] cingulate and medial frontal cortex, hippocampal pyrimidal layer, thalamic basolateral nucleus, lateral septum, colliculi, substantia nigra, and periaqueductal grey.[14] Mu receptors are found in other tissues as well, a notable one being the cerebellum in rabbits.[15]

Table 1. Opioid Ligands Used To Characterize Opioid Receptors[A]

Receptor Type	Agonist	Antagonist
μ	DAMGO	naloxone
	dermorphin[B]	naltrexone
δ	DADLE	ICI 174,864
	DSLET, DTLET	naltrindole
	DPDPE	
	deltorphin[B]	
κ	bremazocine	Nor-BNI
	U 50,488, U 69,593	

[A] All compounds in Table 1 are from ref. 10 unless otherwise indicated.

[B] From ref. 11.

Delta receptors, likewise, are found in several areas of the CNS including the caudate nucleus,[12] and layers I, II, V, and VI of the cerebral cortex, neocortex, olfactory bulb, N. accumbens, amygdala, and striatum.[13] There is a lack of δ receptors in the midbrain, hindbrain, and thalamic nuclei.[14]

In both rat and guinea pig brain κ receptors are found in layers V and VI of the cerebral cortex,[12] olfactory bulb, N. accumbens, striatum, and substantia nigra.[14] In guinea pig κ receptors are also found in the pyriform cortex and low levels in the thalamic and hypothalamic nuclei.[14] The opioid receptors in the guinea pig cerebellum are almost all of the κ type.[16] In rat brain κ receptors are found in relatively low levels in the central grey and the thalamic and hypothalamic nuclei.[14] Kappa receptors are found in relatively high levels in the spinal cord[17] and human placenta.[18,19]

Of pharmacological importance, all three receptor types are found in the mouse vas deferens, and μ and κ receptors are located in the guinea pig myenteric plexus (guinea pig ileum - GPI).[20] Opioid receptors in the rabbit and hamster vas deferens are almost all of the κ[21] and δ[22] types, respectively.

Actions of Opioid Agonists

The most notable action of opioid drugs in humans at any receptor type is analgesia. At the μ receptor, morphine and other μ agonists produce analgesia at supraspinal sites. Other effects of μ receptor activation include reduced gonadotropin and testosterone levels,[23] respiratory and cardiovascular depression, miosis, reduced gastrointestinal (GI) motility, euphoria, and high abuse potential.[24] Kappa selective agonists act in the spinal cord to produce analgesia and antinociception for long term inflammatory pain[25] and nonthermal noxious stimuli,[26] with less respiratory depression, constipation, miosis, and abuse liability compared to μ agonists.[27] However, κ-mediated action produces dysphoric, psychotomimetic effects rather than euphoria.[27] The results of δ

receptor activation are not well defined in humans because the specific δ ligands do not cross the blood-brain barrier. Analgesia and positive reinforcing effects at supraspinal sites, antinociception for thermal stimuli at spinal sites, and cardiovascular depression are the effects caused by specific δ agonists administered in animals.[28,29]

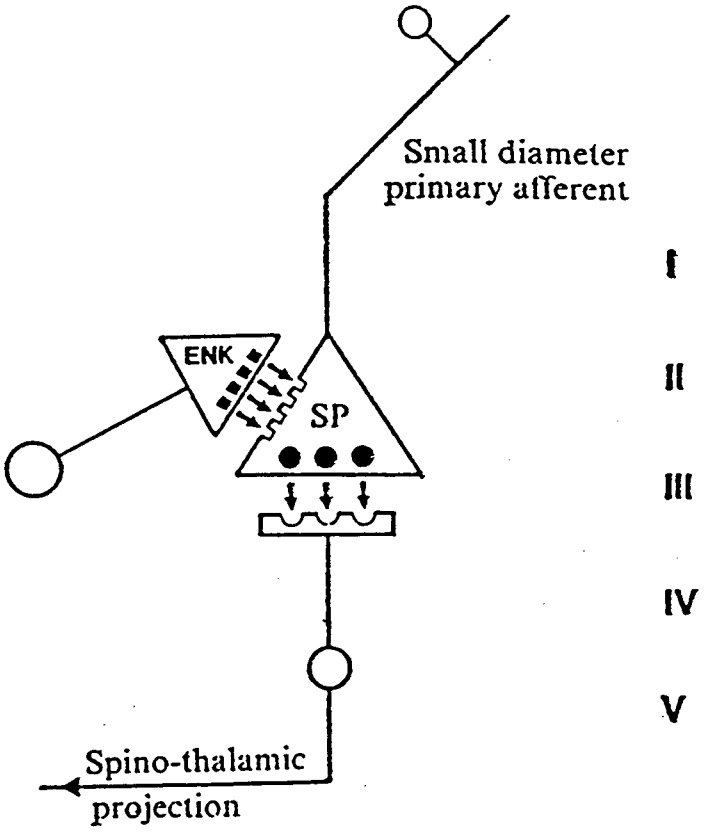
Mechanisms of Action

It has been proposed that opioids acting on all three receptor types have two distinct biochemical actions. First, when the receptors are located at the presynaptic area of the nerve, opioids act as neuromodulators, resulting in decreased release of excitatory neurotransmitters (see Fig. 1).[1] Mu receptor activation in rat brain cortical slices inhibits norepinephrine[30] and acetylcholine turnover,[31] while in rat brain striatal slices activation of κ receptors inhibits dopamine release and δ receptor activation inhibits acetylcholine release.[30]

In rats, μ, κ, and δ receptors also act as neuromodulators in the hypothalamic-pituitary-adrenal axis to modulate an increase in the anterior pituitary hormones prolactin,[32] growth hormone,[32] corticosterone,[33,34] and ACTH,[33,34] and a decrease in thyroid stimulating hormone (TSH), the gonadotrophins, and the posterior pituitary hormones vasopressin and oxytocin.[32] In humans the actions of exogenously administered opioids are the same, except that the level of TSH is elevated.[32]

Second, when the receptors are located postsynaptically, activation of μ and δ receptors inhibits adenylate cyclase.[1,30] This may lead to increased outward K^+ conductance through Ca^{2+}-activated K^+ channels resulting in hyperpolarization of the cell and a decrease in Ca^{2+} flux.[1,9] Activation of κ receptors leads directly to a reduction of Ca^{2+} conductance due to the closing of voltage-dependent N-type Ca^{2+} channels.[1,2] G-proteins may be involved in the coupling of opioid binding sites to ion channels; the hydrolysis of phosphoinositides is another possibility.[2]

Fig. 1 Schematic Representation of a Possible Mechanism for Opiate-Induced Suppression of Substance P (SP) Release.



SP is shown localized within athe terminal of a small- diameter afferent fiber which forms an excitatory axodendritic synapse with the process of a spinal cord neuron originating in lamina IV or V and projecting rostrally. A local enkephalin-containing inhibitory interneuron (ENK), confined to laminae II and III, forms a presynaptic contact on the terminal of the primary afferent. Opioid receptors are depicted presynaptically. From Simon, E. J. *Med. Res. Rev.* **1991**, *11*, 357-374.

Opioid Peptides

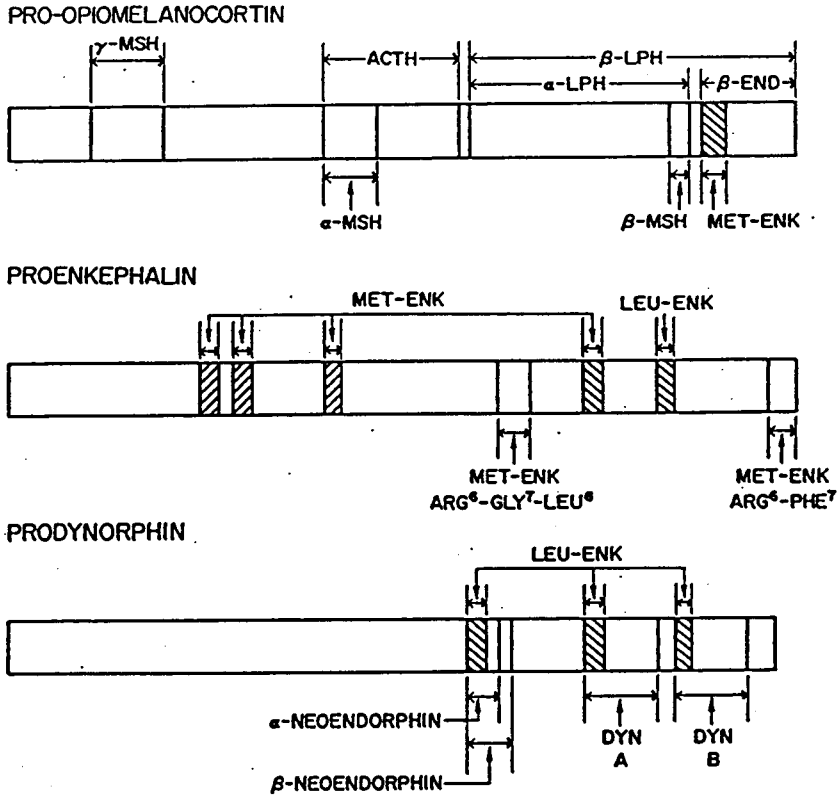
Endogenous Opioid Peptides

There are three families of peptides that have been isolated, characterized, and identified as endogenous opioid peptides in mammals: the enkephalins, endorphins, and dynorphins. These are derived from the proteolytic cleavage of the precursor proteins proopiomelanocortin (POMC), proenkephalin, and prodynorphin[9] (see Fig. 2).[2] POMC is formed mainly in the pituitary and its primary opioid product is β-endorphin, which is located in the pituitary with smaller amounts in the hypothalamus, pancreas, gastric antrum, and the placenta.[35,36] β-Endorphin is equipotent at both δ and μ receptors.[37] POMC contains many nonopioid peptides including ACTH as well. Proenkephalin, formed in the adrenal medulla and striatum,[38] contains four copies of Met-enkephalin, two copies of extended Met-enkephalin, and one copy of Leu-enkephalin,[1] all of which show a high preference for δ receptors. The enkephalins are located in various sections of the brain, autonomic ganglia, GI tract, reproductive organs, and adrenal medulla.[9] Prodynorphin, isolated from brain, spinal cord, pituitary, adrenals, and reproductive organs,[1] is the source of dynorphin A (Dyn A) and its 1-8 and 1-9 fragments, which act at κ receptors.[39] These peptides are located primarily in the spinal cord, parts of the brain, and the GI tract.[37] Other opioid peptides formed from prodynorphin are α- and β-neoendorphin and Dyn B.[1]

Dynorphin A and Structure-Activity Relationship Studies

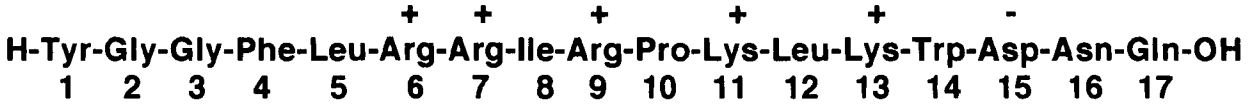
Dynorphin A (Dyn A) was first identified and isolated from porcine pituitary.[40] It is a potent opioid peptide that interacts preferentially with κ opioid receptors, and thus Dyn A has been postulated to be an endogenous κ ligand.[41] Dyn A is a 17-amino acid peptide (Fig. 3) with two distinct regions. The N-terminal tetrapeptide "message" is identical to that of other mammalian opioid peptides, while the

Fig. 2 Schematic Representation of the Structures of the Precursors of the Three Families of Opioid Peptides.



Abbreviations: ENK = enkephalin; DYN = dynorphin; END = endophin. Other abbreviations are defined in the text. The sequence of met-enkephalin is Tyr-Gly-Gly-Phe-Met, while that of leu-enkephalin is Tyr-Gly-Gly-Phe-Leu. From Jaffe, J.H.; Martin, W. R. In *Goodman and Gilman's: The Pharmacological Basis of Therapeutics*, 8th ed., Gilman, A. G., Rall, T. W., Nies, A. S., Taylor, P., Eds.; Pergamon Press, New York, 1990; pp 485-521.

Fig. 3 Dynorphin A



C-terminal "address" is unique to Dyn A.[42] A few studies have been done to determine the importance of individual residues for opioid potency and receptor selectivity (see Chapter I).

Many attempts have been made to determine the structural conformation of Dyn A using physical methods such as NMR, IR, Raman, and fluorescent spectroscopies in different media (see Chapter II),[43-50] but no one conformation can be agreed upon. Because opioid receptors are not present in these physical measurement experiments, the biologically active conformation of Dyn A may not be found by these means. Another method to determine biologically active conformations of a peptide is to prepare conformationally constrained analogues. A region of the peptide is modified in order to disallow specific bond rotations and constrain the peptide to a limited number of possible conformations. Common constraints involve replacing sections of the peptide backbone with rigid entities or cyclizing from side-chain to side-chain, side-chain to backbone, side-chain to terminus, or terminus to backbone. The first analogues of Dyn A constrained in the "message" sequence, cyclo[D-Cys^2,Cys^5]Dyn A-(1-13)[51] and cyclo[D-Orn^2,Asp^5]-Dyn A-(1-8),[52] show high potency in the GPI, but also exhibit high μ-receptor affinity. The other reported cyclic analogues of Dyn A, cyclized via a lactam, cyclo[Orn^5,Asp^8]-, cyclo[Orn^5,Asp^{10}]-, and cyclo[Orn^5,Asp^{13}]Dyn A-(1-13),[52] or via a disulfide, cyclo[Cys^5,Cys^{11}]-, and cyclo[Cys^5,Cys^{11},D-Ala^8]Dyn A-(1-11)NH_2, and cyclo[Cys^8,Cys^{13}]-, cyclo[D-Cys^8,Cys^{13}]-, and cyclo[D-Cys^8,D-Cys^{13}]Dyn A-(1-13)NH_2,[53] are constrained in the C-terminal "address" portion of the peptide. The cyclic lactam derivatives are all selective for μ-opioid receptors, while the cyclic disulfide peptides have high binding affinities for both μ and κ receptors. The only reported Dyn A analogues conformationally constrained in the "message" sequence with selectivity for κ receptors are described in Chapter II.

Solid-Phase Peptide Synthesis[54]

Solid-phase peptide synthesis (see Fig. 4), devised by R.B. Merrifield in 1963,[55] revolutionized peptide chemistry by eliminating the necessary isolation of peptide products and most of the solubility problems found with solution synthesis. In solid-phase synthesis, the growing peptide chain is attached to an insoluble support resin which can easily be rinsed and filtered free of soluble by-products and excess reagents while retaining the peptide. A resin consists of a polymeric solid support which is functionalized with an anchor group to which a linker specific to the type of chemical protocol used is attached. The resin I used in synthesis was the PAL® (peptide amide linker) resin which consists of a polystyrene support that is functionalized with a methylbenzhydryl amine (MBHA) anchor with the 9-fluorenylmethyloxycarbonyl (Fmoc)-protected PAL® linker attached (see Fig. 5). The peptide is removed from the PAL resin with trifluoroacetic acid (TFA) to give the peptide amide. The peptide amide is prepared because it is more stable in biological assays to both endo- and exopeptidase enzymatic degradation than the free acid.

The amino acids used in peptide synthesis must have the amino group and reactive side-chain groups protected so that no undesired reactions occur as the amino acids are coupled sequentially to the growing peptide chain. The first chemical methodology for protection during solid-phase synthesis was the tert-butyloxycarbonyl (Boc) protocol. The Boc group protects the amine group of the amino acid being coupled at its carboxyl end to the growing peptide. The Boc group is removed with a mild to moderate acid (HCl in acetic acid or TFA) before coupling of the next amino acid. Therefore, the reactive side chains of the amino acids must be protected with much more acid-stable groups. Amine, acid, or hydroxyl functions are protected via a benzyl ester or benzyl ether, while the other reactive functions require more specialized protecting groups. To form the new peptide bond, the coupling agents dicyclohexane-

Fig. 4 Solid-Phase Peptide Synthesis

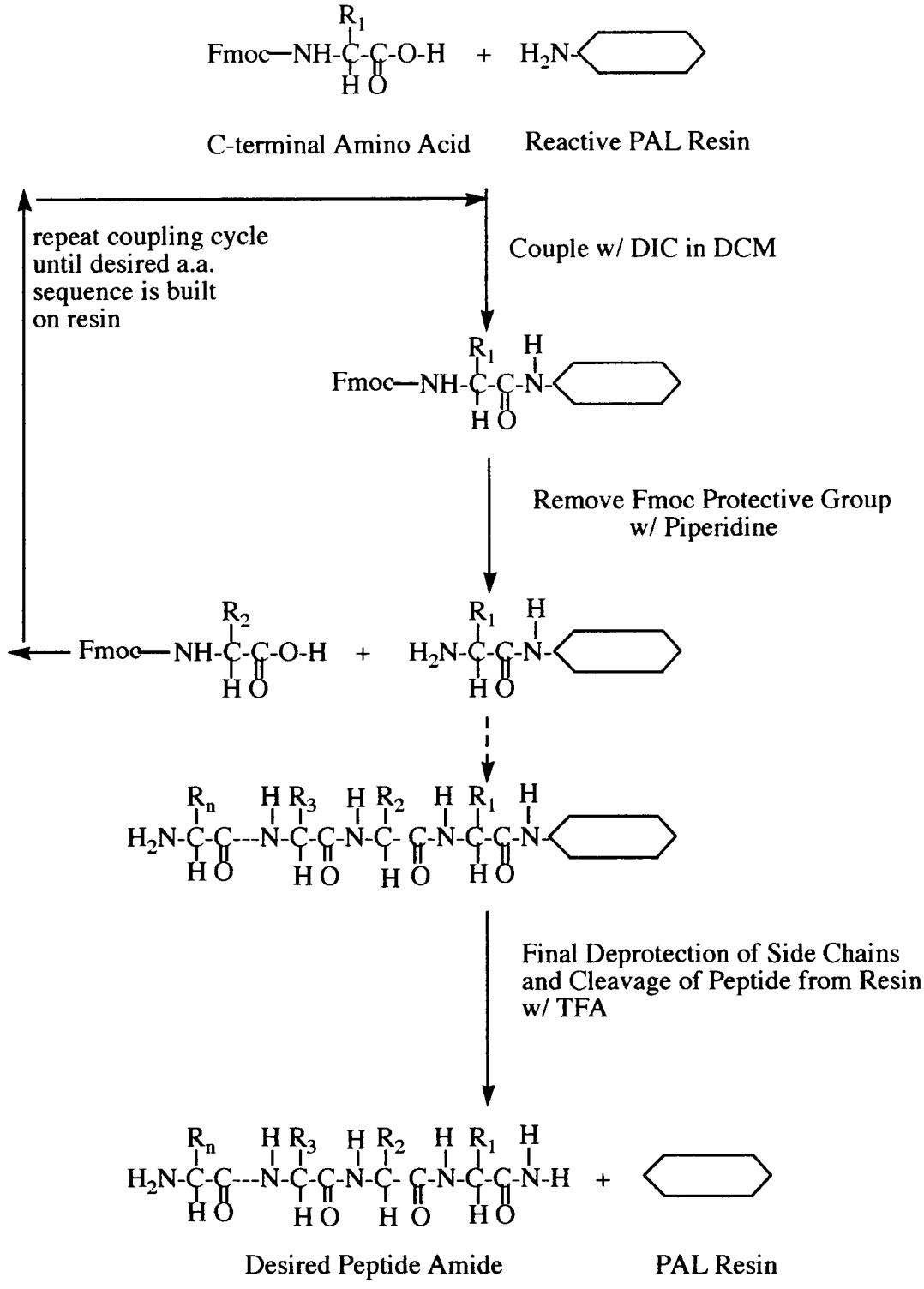
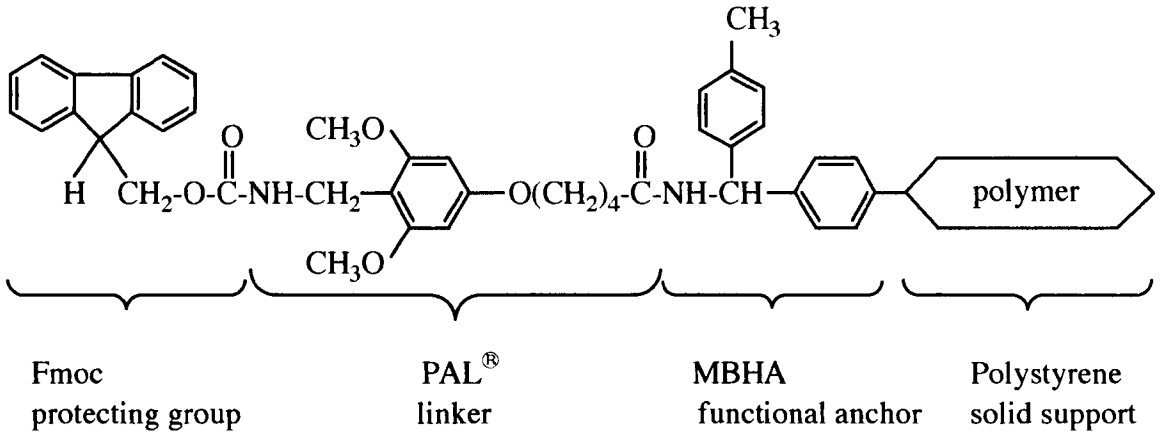


Fig. 5 Fmoc-PAL® Resin



or diisopropylcarbodiimide are often used, although there are other methods including several newer ones. When the desired peptide chain is complete, it is normally cleaved from the resin, with a strong acid (liquid HF or HBr in acetic acid), and the side chain protecting groups removed at the same time. The disadvantages of this synthetic strategy stem from the necessity of using weak acid for the α-amino deprotection and a very strong acid for the final deprotection and cleavage. The weaker acid used to cleave the Boc group may cause premature deprotection of a small percentage of the side chains and some cleavage from the resin, resulting in unwanted side products. The strong acids are not only unpleasant and dangerous to handle, but they require special apparatus, and cause undesirable side reactions at specific amino acids.

The Fmoc protocol for solid-phase synthesis has gained in popularity over the Boc chemistry because it has eliminated some of the undesirable problems found in the latter. A strategy of orthogonal protection, the Fmoc protocol allows the amino protecting group (Fmoc) to be removed by weak organic base (piperidine) in preparation for coupling to the next amino acid, while the side-chain protecting groups remain in place. Side chain amines, acids, and hydroxyls are protected by t-butyl derivatives to form acid-labile urethane, ester, and ether linkages, respectively, while other reactive side chains are protected by specialized protecting groups such as 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) used for Arg (see Chaps. I and II). The coupling agents used are the same as in the Boc protocol. After the peptide with the desired amino acid sequence is complete, it is cleaved from the resin by acid (TFA); the side-chain protecting groups are also removed in this step. Although there are great advantages of orthogonal protection there are still side reactions specific to some amino acids that one must be aware of. Using a scavenger cocktail during cleavage can keep these to a minimum.

Chapter I

Effect of Modification of the Basic Residues of Dynorphin A-(1-13) Amide on Kappa Opioid Receptor Selectivity and Opioid Activity§Δ

Kristin R. Snyder,+ Sandra C. Story,+ Mia E. Heidt,+ Thomas F. Murray,+Gary E. DeLander,+ and Jane V. Aldrich+#*

+College of Pharmacy and #Center for Gene Research and Biotechnology, Oregon State University, Corvallis OR 97331

(* to whom correspondence should be addressed)

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Δ Abbreviations

Abbreviations used for amino acids follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977-983. Amino acids are in the L-configuration. Additional abbreviations used are as follows: Boc, *tert*-butyloxycarbonyl; DAMGO, [D-Ala^2,MePhe^4,Glyol]enkephalin; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DMA, N,N-dimethylacetamide; DMF, N,N-dimethylformamide; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; Dyn A, dynorphin A; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; Lys(Ac), N$^{\varepsilon}$-acetyllysine; MBHA, 4methylbenzhydryl-amine; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; TFA, trifluoroacetic acid.

Abstract

A series of dynorphin A-(1-13) amide (Dyn A (1-13)NH_2) analogues containing lysine or N^{ε}-acetyllysine (Lys(Ac)) was prepared by solid-phase peptide synthesis and evaluated for opioid receptor affinity in radioligand binding assays and for opioid activity in the guinea pig ileum (GPI). Substitutions were made at positions 6, 7, 9, 11, and 13, the basic amino acids in the C-terminus of the peptide, in order to assess the individual contributions of these residues to the κ opioid receptor affinity and selectivity of Dyn A-(1-13)NH_2. While substitutions of Lys(Ac) for Arg in position 6 did not affect κ receptor affinity, it enhanced affinity for μ and δ receptors and therefore caused a loss of κ receptor selectivity. When Lys(Ac) was substituted for Arg^9, κ opioid receptor affinity was enhanced and κ receptor selectivity was retained. Replacement of Arg^7, Lys^{11}, or Lys^{13} by Lys(Ac) resulted in both decreased affinity and selectivity for κ receptors. These results demonstrate the importance of Arg^6 to the receptor selectivity profile of Dyn A-(1-13)NH_2 and indicate that of the five basic residues in the C-terminus, only Arg^9 can be replaced by a nonbasic residue without substantial loss of κ opioid receptor selectivity.

Introduction

Dynorphin A (Dyn A), originally isolated from porcine pituitary,[40] is postulated to be an endogenous κ opioid receptor ligand.[42] Dyn A shares a common N-terminal tetrapeptide sequence (the "message" sequence) with other mammalian opioid peptides, while containing a unique C-terminal "address" sequence which imparts selectivity for κ opioid receptors.[41] The shortened Dyn A-(1-13), with the sequence Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys, accounts for essentially all of the biological activity of Dyn A in the guinea pig ileum (GPI) assay.[56]

Shortly after the determination of the sequence of Dyn A-(1-13), studies were undertaken to determine which amino acids in the C-terminal "address" region of the peptide are important for opioid potency and κ receptor selectivity. The first of these involved successive truncation of Dyn A-(1-13) from the C-terminus and the evaluation of the fragments in the GPI.[41] This study found that the basic residues Arg^{7}, Lys^{11}, and Lys^{13} were important residues for potency. Based upon the sensitivity of the fragments to antagonism by naloxone, it was concluded that Arg^{7} and Lys^{11} made the greatest contributions to κ receptor selectivity. In another study, Dyn A and related fragments Dyn A-(1-13), -(1-11), -(1-9), -(1-7), and -(1-5) were tested in ileum preparations made tolerant to morphine or ethylketocyclazocine.[57] While the extension of Leu-enkephalin by the two basic residues in positions 6 and 7 conferred some dynorphin-like properties to the peptide, the longer fragments Dyn A-(1-11) and Dyn A-(1-13) exhibited the greatest preference for κ receptors. Because fragments were used in both of these studies, however, the contributions of individual C-terminal residues to κ opioid receptor selectivity and potency could not be separated from the effects of previously truncated residues. In particular, the contribution of Arg^{6} to κ receptor selectivity could not be determined, since truncation of the important Arg^{7} residue results in a peptide, Dyn A-(1-6), which no longer exhibits selectivity for κ receptors.

The contribution of individual residues to potency and receptor selectivity can be assessed by amino acid substitution in the peptide. In one study, alanine was systematically substituted for each amino acid in positions 1 through 11 in Dyn A-(1-13).[58] The analogues were evaluated for opioid activity in smooth muscle assays and for opioid receptor affinity in a [^{3}H]etorphine binding assay in rat brain. From this study the most important sites in the "address" portion for potency appeared to be Arg^6 and Arg^7, with Arg^9 and Lys^{11} making smaller contributions. No information was reported, however, on what effects these substitutions had on selectivity for κ receptors.

We are interested in preparing a variety of Dyn A analogues, including conformationally constrained derivatives, with enhanced selectivity for κ opioid receptors. While the contributions of Arg^7 and Lys^{11} to Dyn A's potency and selectivity are generally accepted, the relative importance of the basic residues in positions 6 and 9 are less clear. Thus these latter two positions could be potential sites for incorporation into a conformational constraint or other structural modification. Incorporation into a conformational constraint such as a lactam or cyclic disulfide would entail the loss of the residue's basicity. Therefore we investigated whether either of these positions could be replaced by a nonbasic residue by preparing linear analogues containing N$^\varepsilon$-acetyllysine (Lys(Ac)) in each of these positions. We also substituted Lys(Ac) for the basic residues in positions 7, 11 and 13 in Dyn A-(1-13)NH_2. This allowed us to evaluate the relative importance for κ opioid receptor selectivity and potency of residues 6 and 9 as compared to residues 7, 11 and 13. We used the peptide amide because Dyn A-(1-13)NH_2 retains the κ receptor selectivity of the corresponding acid and is more stable to enzymatic degradation.[59] Since arginine is normally present in positions 6, 7 and 9 in Dyn A-(1-13), the analogues containing lysine in these positions were also prepared to allow for a more direct comparison to the acetylated analogues.

Results

Peptide Synthesis and Purification. All peptides were synthesized on a PAL resin (a 4-methylbenzhydrylamine (MBHA) resin with a 5-[4-(aminomethyl)-3,5-dimethoxyphenoxy]-valeric acid linker[60]) using (9-fluorenylmethoxy)carbonyl (Fmoc) protected amino acids. Side-chain protecting groups used in this synthetic strategy were *tert*-butyloxycarbonyl (Boc) for Lys, 2,2,5,7,8-pentamethylchromanyl-6-sulfonyl (Pmc)[61] for Arg, and *tert*-butyl (tBu) for Tyr. The Fmoc-protected amino acids were coupled to the growing peptide chain using diisopropyl-carbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt) in DCM/DMF or DCM/DMA (1/1). The Fmoc protecting group was removed with 30% piperidine in DMF/toluene or DMA/toluene (1/1). The peptides were deprotected and cleaved from the PAL resin using concentrated trifluoroacetic acid (TFA) in the presence of scavengers (generally Reagent K[62]) and purified by preparative reverse phase HPLC. The purified peptides were characterized by analytical HPLC, fast atom bombardment mass spectrometry (FAB-MS), and amino acid analysis (Tables 2 and 3).

Opioid Receptor Binding Affinities and Opioid Activity. The peptides were evaluated for opioid receptor affinity at κ, μ, and δ receptors by measuring the inhibition of binding of [^{3}H]bremazocine, [^{3}H]DAMGO ([D-Ala^2,MePhe^4,Glyol]-enkephalin),[63] and [^{3}H]DPDPE ([D-Pen^2,D-Pen^5]enkephalin),[64,65] respectively (Table 4), as previously described.[66] Kappa opioid receptor affinity was determined in the guinea pig cerebellum, since over 80% of the opioid receptors in this tissue are κ receptors.[16] Mu and δ opioid receptor affinities were assessed in rat forebrain membranes. The opioid activity of the peptides was determined in the electrically-stimulated GPI[66] (Table 5).

Table 2. Yields, HPLC Analysis, and Fast-Atom Bombardment Mass Spectrometry (FAB-MS) Analysis of [Lys]- and [Lys(Ac)]Dyn A-(1-13)NH_2 Analogues

Dyn A-(1-13)NH_2 Analogue	% Yield	HPLC Gradient[a] R_v in mL	HPLC Isocratic R_v in mL (%B)[b]	FAB-MS M+1
Lys^6	37	31.4	19.8 (16%)	1575
$Lys(Ac)^6$	45	30.0	16.8 (16%)	1617
Lys^7	8[c]	29.4	11.9 (17%)	1575
$Lys(Ac)^7$	49	30.2	11.2 (19%)	1617
Lys^9	81	27.9	16.2 (18%)	1575
$Lys(Ac)^9$	56	30.5	22.2 (18%)	1617
$Lys(Ac)^{11}$	26	30.6	7.5 (15%)	1645
$Lys(Ac)^{13}$	40	30.1	11.9 (19%)	1645

[a] 0 to 75% B over 50 min at 1.5mL/min.
[b] solvent composition indicated in parentheses
[c] yield low due to procedural losses during purification - see experimental section

Table 3. Amino Acid Analysis of [Lys]- and [Lys(Ac)]-Dyn A-(1-13)NH_2 Analogues

Analogue	Y(1)	G(2)	F(1)	L(2)	R(2/3)	I(1)	P(1)	K(2/3)
Lys^6	0.95	2.00	0.98	2.03	2.10	0.93	1.03	3.01
$Lys(Ac)^6$	0.86	2.03	0.96	1.96	2.12	0.86	0.21	3.02
Lys^7	0.88	1.94	0.96	2.10	2.01	1.08	1.18	2.85
$Lys(Ac)^7$	0.99	1.97	0.98	1.97	2.07	0.95	1.12	2.97
Lys^9	0.89	2.03	1.00	2.14	2.04	1.07	1.06	2.78
$Lys(Ac)^9$	0.87	2.06	0.98	2.14	2.04	1.04	1.09	2.79
$Lys(Ac)^{11}$	0.89	2.06	0.99	2.18	2.94	1.00	1.04	1.91
$Lys(Ac)^{13}$	1.02	1.96	1.01	1.98	3.07	0.93	1.02	1.99

Table 4. Opioid Receptor Binding Affinities and Selectivities of [Lys]- and [Lys(Ac)]Dyn A-(1-13)NH_2 Analogues

	K_i (nM)			
Peptide	**[^{3}H]Bremazocine**	**[^{3}H]DAMGO**	**[^{3}H]DPDPE**	**Receptor Selectivity (κ/μ/δ)[a]**
Dyn A-(1-13)NH_2	0.023 ±0.009	0.40 ± 0.06	1.9 ± 0.2	1 / 18 / 83
Lys^6	0.020 ± 0.005	0.075 ± 0.018	5.9 ± 1.7	1 / 3.9 / 300
$Lys(Ac)^6$	0.024 ± 0.003	0.018 ± 0.003	0.42 ± 0.11	1.3 / 1 / 23
Lys^7	0.021 ± 0.001	0.055 ± 0.006	1.6 ± 1.4	1 / 2.6 / 78
$Lys(Ac)^7$	0.14 ± 0.06	0.18 ± 0.02	1.1 ± 0.2	1 / 1.3 / 7.7
Lys^9	0.0067 ± 0.0010	0.046 ± 0.005	1.9 ± 0.4	1 / 6.9 / 400
$Lys(Ac)^9$	0.0042 ± 0.0013	0.043 ± 0.008	1.3 ± 0.2	1 / 10.4 / 490
$Lys(Ac)^{11}$	0.053 ± 0.017	0.030 ± 0.001	0.45 ± 0.27	1.8 / 1 / 15
$Lys(Ac)^{13}$	0.15 ± 0.06	0.061 ± 0.004	4.6 ± 0.7	2.4 / 1 / 75

[a] Ratio of K_i's where the lowest K_i is used as the denominator

Table 5. Opioid Activity of [Lys]- and [Lys(Ac)]Dyn A(1-13)NH_2 Analogues in the Guinea Pig Ileum

Dyn A(1-13)NH_2 Analogue	IC_{50} in nM (95% Confidence Interval)	Relative Potency (%)
Dyn A-(1-13)NH_2	0.24 (0.21-0.29)	100
Lys^6	0.25 (0.13-0.50)	94
$Lys(Ac)^6$	0.64 (0.31-1.34)	37
Lys^7	0.19 (0.13-0.29)	122
$Lys(Ac)^7$	0.34 (0.26-0.45)	69
Lys^9	0.27 (0.19-0.37)	89
$Lys(Ac)^9$	0.26 (0.18-0.38)	91
$Lys(Ac)^{11}$	0.16 (0.12-0.21)	148
$Lys(Ac)^{13}$	0.19 (0.14-0.24)	126

Replacement of the arginines at positions 6,7 and 9 in Dyn A-(1-13)NH_2 by lysine generally caused the greatest changes in receptor affinity at μ receptors, where receptor affinity was enhanced 5- to 22-fold compared to Dyn A-(1-13)NH_2 (Table 4). The affinity for κ receptors was unchanged when the arginines at positions 6 and 7 were replaced by lysine, while substitution of lysine for Arg^9 increased κ receptor affinity 3-fold when compared to the parent peptide. The net result was a loss of discrimination between κ and μ receptor binding for all three of the analogues. The substitution of lysine in positions 7 and 9 had little effect on the analogues' affinity for δ receptors, while [Lys^6]Dyn A-(1-13)NH_2 exhibited decreased affinity for δ opioid receptors. [Lys^7]Dyn A-(1-13)NH_2 thus showed discrimination between κ and δ opioid receptors similar to that of the parent peptide, while [Lys^6]- and [Lys^9]Dyn A-(1-13)NH_2 showed improved discrimination between κ and δ receptors compared to Dyn A-(1-13)NH_2.

The positive charge at each of positions 6, 7, 9, 11, and 13 was removed by acetylating the side-chain amine of lysine. Unlike replacement of Arg^6 by Ala, which decreases opioid receptor affinity in rat brain,[58] acetylation of lysine in position 6 had little effect on κ receptor affinity. It enhanced μ binding affinity, however, causing a complete loss of κ receptor selectivity. When the 7 position was acetylated, κ binding showed a 6-fold decrease while μ binding decreased only 3-fold, so discrimination between the two receptor types was again nonexistent. Binding affinity for κ receptors decreased 2-fold and 6-fold for [Lys(Ac)^{11}]- and [Lys(Ac)^{13}]Dyn A-(1-13)NH_2, respectively, while the affinity of these peptides for μ receptors increased 6- to 13-fold, so that they exhibited a slight preference for μ over κ receptors. The one exception to this loss of κ selectivity was [Lys(Ac)^9]Dyn A-(1-13)NH_2, which showed enhanced κ receptor affinity and selectivity compared to its parent, [Lys^9]Dyn A-(1-13)NH_2, and was the most κ selective of the Dyn A-(1-13)NH_2 analogues prepared. The marked enhancement in affinity for κ and μ receptors by [Lys(Ac)^9]Dyn A-(1-13)NH_2 over Dyn

A-(1-13)NH_2 is in contrast to the modest decrease in affinity observed for [Ala^9]Dyn A-(1-13) in [^{3}H]etorphine binding assay in rat brain.[58]

Acetylation in the 6, 7 and 11 positions enhanced affinity for δ receptors, and thus decreased the discrimination between κ and δ receptors for these peptides. Interestingly, acetylation of Lys^{13} decreased affinity for δ opioid receptors, but because of this analogue's decreased affinity for κ receptors its discrimination between κ and δ receptors was very similar to that of Dyn A-(1-13)NH_2. While acetylation at the 9 position slightly increased δ opioid receptor affinity, the marked enhancement in the κ receptor affinity of [Lys(Ac)^9]Dyn A-(1-13)NH_2 increased its discrimination for κ over δ receptors, similar to its parent peptide [Lys^9]Dyn A-(1-13)NH_2.

All of the analogues exhibited subnanomolar potency in the guinea pig ileum (Table 5). Only [Lys(Ac)^6]Dyn A-(1-13)NH_2 showed a significant decrease in opioid activity when compared to either its parent peptide [Lys^6]Dyn A-(1-13)NH_2 or Dyn A-(1-13)NH_2. [Lys^9]- and [Lys(Ac)^9]Dyn A-(1-13)NH_2, which were very potent analogues in the κ and μ binding assays, exhibited potency similar to Dyn A-(1-13)NH_2 in the GPI. Overall, the replacement of the basic residues by Lys(Ac) was better tolerated than replacement by Ala, which caused at least a 7-fold decrease in activity in the GPI.[58] The antagonism of selected analogues by norbinaltorphimine, a κ-selective antagonist,[67,68] was examined in order to evaluate whether these peptides were acting on κ receptors in the GPI. Norbinaltorphimine at a dose of 10 nM consistently shifted the dose-response curves of [Lys^6]-, [Lys(Ac)^6]-, [Lys^9]-, [Lys(Ac)^9]Dyn A-(1-13)NH_2 and Dyn A-(1-13)NH_2 20- to 30-fold, indicating that the agonist activity of these compounds in the GPI is mediated at least in part by κ receptors.

Conclusions and Discussion

The above results have clear implications for the design of future Dyn A analogues, particularly the design of conformationally constrained peptides. Our results for [Lys(Ac)^6]Dyn A-(1-13)NH_2 demonstrate that Arg^6 is a key determinant in the "address" sequence of Dyn A for the peptide's receptor selectivity profile. As mentioned above, the importance of this residue was not revealed by the earlier studies of truncated sequences.[57,58] While a basic residue at position 6 in Dyn A is not required for κ receptor binding, it decreases the peptide's interactions with μ and δ receptors, enhancing κ selectivity. These results suggest that cyclization through this position to give conformationally constrained analogues could have an adverse affect on selectivity for κ receptors. Of the five basic residues in the "address" sequence, our data indicate that only Arg^9 can easily be replaced by a nonbasic amino acid without substantial loss of κ receptor selectivity, making this is a logical site for incorporation into a conformational constraint. Our results for substitution of the remaining basic residues in positions 7, 11, and 13 are consistent with the earlier observations that these residues are important for κ receptor affinity and selectivity.[57]

Experimental Section

Materials. The reagents used in peptide synthesis were as follows: FmocPAL Resin (MBHA resin with a 5-[4-(Fmoc-aminomethyl)-3,5-dimethoxyphenoxy]valeric acid linker), HOBt and all amino acids except FmocLys and FmocLys(Ac) (Milligen/Biosearch, Novato, CA); FmocLys (Bachem, Torrence, CA); TFA (Kali-Chemie, Greenwich, CT); anisole, ethanedithiol, and DIC (Aldrich); acetic anhydride (Baker); and HPLC-grade solvents DCM, DMF, DMA, MeOH (Burdick and Jackson or Merck Omnisolv). The reagents and supplies used in purification were as follows: HPLC-grade AcCN and MeOH (Burdick and Jackson); TFA (Pierce Sequanal grade in

amber ampules); HPLC-grade water (Milli-Q system, College of Pharmacy); Syrfil disposable HPLC filters, 0.45 μm pore size. Other reagents were reagent grade.

FmocLys(Ac) was prepared initially by acetylation of FmocLys with acetic anhydride (6 equiv) in DMF for 3 h, followed by evaporation, trituration with ether, and recrystallization from EtOAc. Subsequently FmocLys(Ac) was obtained from Peninsula Laboratories, Belmont, CA. The melting points (156-158° C) and analytical HPLC (R_v = 39.5 mL, 0 to 75% B over 50 min) were identical for the two samples.

The peptides were synthesized on a Biosearch 9500 automated peptide synthesizer (Novato, CA). The peptides were analyzed using a Beckman Model 431A gradient HPLC system consisting of a Model 421A controller, two Model 110B pumps, Model 201A injector, Model 163 detector, and a Waters Model 740 data module. The peptides were purified on the above system with an ISCO UA-5 UV detector, or on a Rainin HPLC gradient system with HPLX pumps and the ISCO detector. The HPLC columns used were a Vydac 214TP54 C_4 analytical column (300Å, 5 μm, 4.6 mm x 25 cm) with a Vydac C_4 214TP guard cartridge, and a Protein Plus preparative column (300Å, 10 μm, 21 mm x 25 cm) with a Dynamax (21 mm x 5 cm, C_4, 12 μm) guard cartridge. The peptides were eluted using 0.1% TFA in H_2O (Solvent A) and 0.1% TFA in AcCN (Solvent B). For the analytical and preparative columns the flow rates were 1.5 mL/min. and 10 or 20 mL/ min, respectively, and the eluents were monitored at 214 nm (Beckman Model 163 detector) and 280 nm (ISCO UA-5 detector), respectively. The aqueous solutions were lyophilized on a Thermovac Model FD-6 lyophilizer.

The peptides were characterized by FAB-MS and amino acid analysis. The FAB-MS was performed on a Kratos MS50RF in the Department of Agricultural Chemistry at Oregon State University, Corvallis, OR. Peptide samples were hydrolyzed for 24 h at 110° C with 6 N HCl plus 1% phenol and amino acid content determined by the Center for Gene Research and Biotechnology, Central Services Laboratory, Oregon State

University, on a Beckman System Gold Model 126AA (2 x 250 mm Spherogel column), using ninhydrin detection at 570 nm.

Peptide Synthesis using Fmoc-protected Amino Acids

Solid Phase Peptide Synthesis. All peptides were synthesized using the Fmoc chemical protocol on the Biosearch 9500. The FmocPAL resin (0.5 g, 0.27 mmol/g resin substitution) was washed with 5 x 20 mL of DCM/DMF (1/1) and the synthesis carried out as follows: the deprotection of the resin was performed for 3 min, then 7 min in piperidine/toluene/DMF (30/35/35, v/v), and the resin then washed with DCM/DMF (1/1, 12x). The solution of the desired Fmoc amino acid (0.4 M in DMF, 4.0-fold excess) with 1 equivalent of HOBt was mixed with an equal amount of 0.4 M DIC in DCM and reacted with the resin for 2 h; the resin was then washed with DCM/DMF (1/1, 12x). Amino acids with side chain protecting groups used were FmocArg(Pmc), FmocLys(Boc), and FmocTyr(tBu). Ninhydrin was used on a small sample of resin to determine if couplings were complete. Following a coupling reaction the Fmoc group was removed as described above and the next amino acid coupled to the resin. After completion of the peptide assembly and removal of the Fmoc group from the N-terminal residue, the resin was washed successively with DCM/DMF (1/1), DCM, and MeOH, collected and dried overnight *in vacuo*. DMA was used in place of DMF in the synthesis of [Lys(Ac)^{7}]- and [Lys(Ac)^{13}]Dyn A-(1-13)NH_2.

Cleavage of the Peptide from the Support. The dried protected peptide resins were generally reacted at room temperature with 5 mL Reagent K[62] (82.5% TFA, 5% water, 5% phenol, 5% thioanisole, and 2.5% ethanedithiol) under nitrogen for 4-5 h. The peptides were filtered from the resin, washed with TFA and concentrated to about 2-5 mL. The solutions were diluted with 10% acetic acid (40 ml)

and then extracted with Et_2O (3x), and the aqueous layers lyophilized to give the crude peptides. [Lys[6]]Dyn A-(1-13)NH_2 was obtained by treating the corresponding resin with 5 mL Reagent R[60] (90% TFA, 5% thioanisole, 3% ethanedithiol, and 2% anisole) under nitrogen for 24 h, followed by filtration, concentration, and trituration of the peptide with ether. [Lys(Ac)[6]]Dyn A-(1-13)NH_2 was cleaved from the resin using TFA/anisole (9/1) overnight, followed by filtration, concentration, and trituration with ether.

Purification of the Peptides. The crude peptides were purified by preparative HPLC using a mobile phase gradient of either 100% A to 50% B over 50 min at 20 ml/min or 100% A to 70% B over 140 min at 10 ml/min. [Lys[7]]Dyn A-(1-13)NH_2 required a second purification by preparative HPLC. Generally peak fractions were analyzed by isocratic analytical HPLC and pure fractions combined and lyophilized. The resulting pure peptides were characterized by FAB-MS, amino acid analysis, and analytical HPLC (Tables 2 and 3).

Binding Assays. Guinea pig cerebellar membranes and rat forebrain membranes were prepared as previously described.[66] (See Appendix A.) The inhibition of the binding of [^{3}H]bremazocine to guinea pig cerebellar membranes (κ) was measured at 4° C in the presence of bestatin as previously reported,[66] except that 100 nM DAMGO was included in the incubation mixtures. Nonspecific binding was determined in the presence of 1 μM Dyn A-(1-13)NH_2. The inhibition of the binding of [^{3}H]DAMGO (μ) and [^{3}H]DPDPE (δ) to rat forebrain membranes was measured at 4° C in the presence of peptidase inhibitors, as previously described;[66] nonspecific binding was measured in the presence of 10 μM levorphanol and 10 μM unlabeled DPDPE, respectively. Equilibrium inhibition constants (K_i's) were calculated from the Cheng

and Prusoff equation,[69] using 0.0549, 0.314 and 7.63 nM for the K_D values of tritiated bremazocine, DAMGO, and DPDPE, respectively.

Guinea Pig Ileum Assay. Guinea pig ileum assays were performed as previously described.[66] (See Appendix A.) In experiments involving nor-binaltorphimine, the antagonist was added to the tissue bath 20 min prior to determination of the agonist dose-response curve.

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Chapter II

Synthesis and Opioid Activity of Dynorphin A-(1-13)NH_2 Analogues Containing *cis*- and *trans*-4-Aminocyclohexanecarboxylic Acid[Δ]

Kristin R. Snyder, Thomas F. Murray, Gary E. DeLander, and Jane V. Aldrich*

Oregon State University, College of Pharmacy, Corvallis, OR 97331

(* to whom correspondence should be addressed)

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Δ Abbreviations

Abbreviations used for amino acids follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature outlined in *J. Biol. Chem.* **1972**, *247*, 977-983. Amino acids are in the L-configuration. Additional abbreviations used are as follows: ACCA, 4-aminocyclohexanecarboxylic acid; Aib, α–aminoisobutyric acid; DAMGO, [D-Ala^2,MePhe^4,Glyol]enkephalin; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; Dyn A, dynorphin A; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; Fmoc-ON-Su, N-9-(fluorenylmethoxycarbonyl)-succinimide; GPI, guinea pig ileum; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

Abstract

It has been proposed that the "message" sequence of dynorphin A (Dyn A) exists in an extended conformation in aqueous solution (Schiller, P.W. *Int. J. Pept. Protein Res.* **1983**, *21*, 307-312). Molecular modeling suggested that *trans*-4-aminocyclohexanecarboxylic acid (*trans*-ACCA) might function as a conformationally constrained replacement for Gly^2-Gly^3 of Dyn A in such an extended conformation. ACCA was synthesized by catalytic hydrogenation of *p*-aminobenzoic acid and the cis and trans isomers were separated by fractional recrystallization. Analogues of Dyn A-(1-13)NH_2 containing *cis*- and *trans*-ACCA were prepared by solid-phase peptide synthesis using the Fmoc chemical protocol. Results from radioligand binding assays indicated that the peptides have modest affinity for κ opioid receptors (K_i's = 9.1 and 13.4 nM for [*cis*-$ACCA^{2-3}$]- and [*trans*-$ACCA^{2-3}$]Dyn A-(1-13)NH_2, respectively) and modest κ-receptor selectivity (K_i ratio (κ/μ/δ) = 1/13/210 and 1/21/103, respectively). [*cis*-$ACCA^{2-3}$]- and [*trans*-$ACCA^{2-3}$]Dyn A-(1-13)NH_2 are the first reported Dyn A analogues constrained in the "message" sequence that are selective for κ receptors. The *cis*-ACCA analogue showed very weak opioid activity (IC_{50} = 4.0 μM) in the guinea pig ileum.

Introduction and Rationale

Dynorphin A (Dyn A), a 17-amino acid peptide, has been postulated to be an endogenous κ opioid receptor ligand.[42] Dyn A shares with other mammalian opioid peptides a common N-terminal tetrapeptide "message" sequence which is important for opioid activity, while containing a unique C-terminal "address" sequence which imparts selectivity for κ opioid receptors.[41] The shortened Dyn A-(1-13), with the sequence Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys, accounts for essentially all of the biological activity of Dyn A in the guinea pig ileum (GPI) assay.[56]

Since Dyn A is a linear peptide, it can adopt a number of possible conformations in solution, with the conformation dependent on the environment. In aqueous solution several spectral techniques, circular dichroism,[43,44] IR spectroscopy,[45,46] Raman spectroscopy,[47] and NMR,[46,48,49] are consistent with a random coil and/or extended β-strand conformation for Dyn A-(1-13). Fluorescent energy transfer experiments with [Trp^4]Dyn A-(1-13) in dilute aqueous solution suggested that the N-terminal portion of Dyn A is in an extended conformation.[50] On the planar surface of a neutral lipid membrane,[45] or in the presence of sodium dodecyl sulfate,[43] however, Dyn A-(1-13) appears to adopt an α-helical structure. Binding to an anionic phospholipid[48] or the addition of cerebroside sulfate[44] does not induce helical structure in the peptide.

The question remains of whether a conformation observed in solution reflects the conformation Dyn A adopts at opioid receptors. Preparing conformationally constrained derivatives is another way to examine the biologically active conformations of a peptide. The two reported analogues of Dyn A constrained in the "message" sequence, cyclo[D-Cys^2,Cys^5]Dyn A-(1-13)[51] and cyclo[D-Orn^2,Asp^5]Dyn A-(1-8),[52] show high potency in the GPI, but also exhibit high μ-receptor affinity. The other reported cyclic analogues of Dyn A, cyclized via either a lactam[52] or a disulfide,[53] are constrained in the C-terminal "address" portion of the peptide.

Since the N-terminal "message" sequence appears to be important for the opioid activity of Dyn A, we incorporated conformational constraints into this region of the peptide. Tyr^1 and Phe^4 residues are important for opioid activity and potency,[41] so conformational constraints involving Gly^2 and/or Gly^3, which would affect the relative orientation of the aromatic rings at positions 1 and 4, were examined. Since several of the conformational studies described above, particularly Schiller's fluorescent energy transfer experiments,[50] suggested an extended conformation for the N-terminus of Dyn A, a conformational constraint consistent with this proposed conformation was chosen for preparation.

Results and Discussion

Design Rationale and Synthesis. Molecular modeling with the AMBER[70,71] program was used to examine possible conformational constraints for incorporation into positions 2 and 3 in Dyn A-(1-13)NH_2. These studies suggested that *trans*-4-aminocyclohexanecarboxylic acid (trans-ACCA) might replace Gly^2-Gly^3 in an extended conformation. The calculated nitrogen-carbonyl carbon distance was 5.70Å for *trans*-ACCA in the diequitorial conformation vs. 6.12Å between the nitrogen of Gly^2 and the carbonyl carbon of Gly^3 when the peptide was in an extended conformation. The ACCA dipeptide replacement is equivalent to constraining ψ_2 and ϕ_3 while still allowing free rotation around ϕ_2 and ψ_3. (Fig. 6.)

ACCA was synthesized by hydrogenation of *p*-aminobenzoic acid with PtO_2 as a catalyst[72] (Fig. 7), which yielded a mixture of cis and trans isomers (approx. ratio of 2.5/1 cis/trans). Separation by fractional recrystallization[73] from EtOH yielded the cis isomer, which was pure by ^{1}H-NMR. Subsequent recrystallization from EtOH/ether yielded the trans isomer, but ^{1}H-NMR indicated that the trans isomer contained from 10-15% up to 35% of the cis isomer, depending on the batch. Following fractional

Fig. 6. Dyn A- and [trans-ACCA$^{2-3}$]Dyn A-(1-13)NH_2

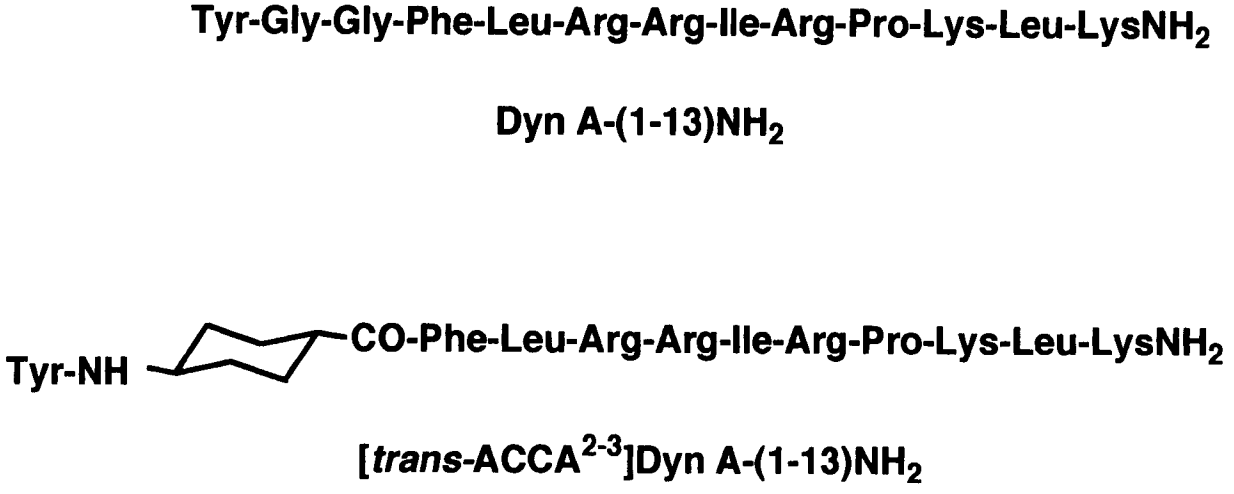
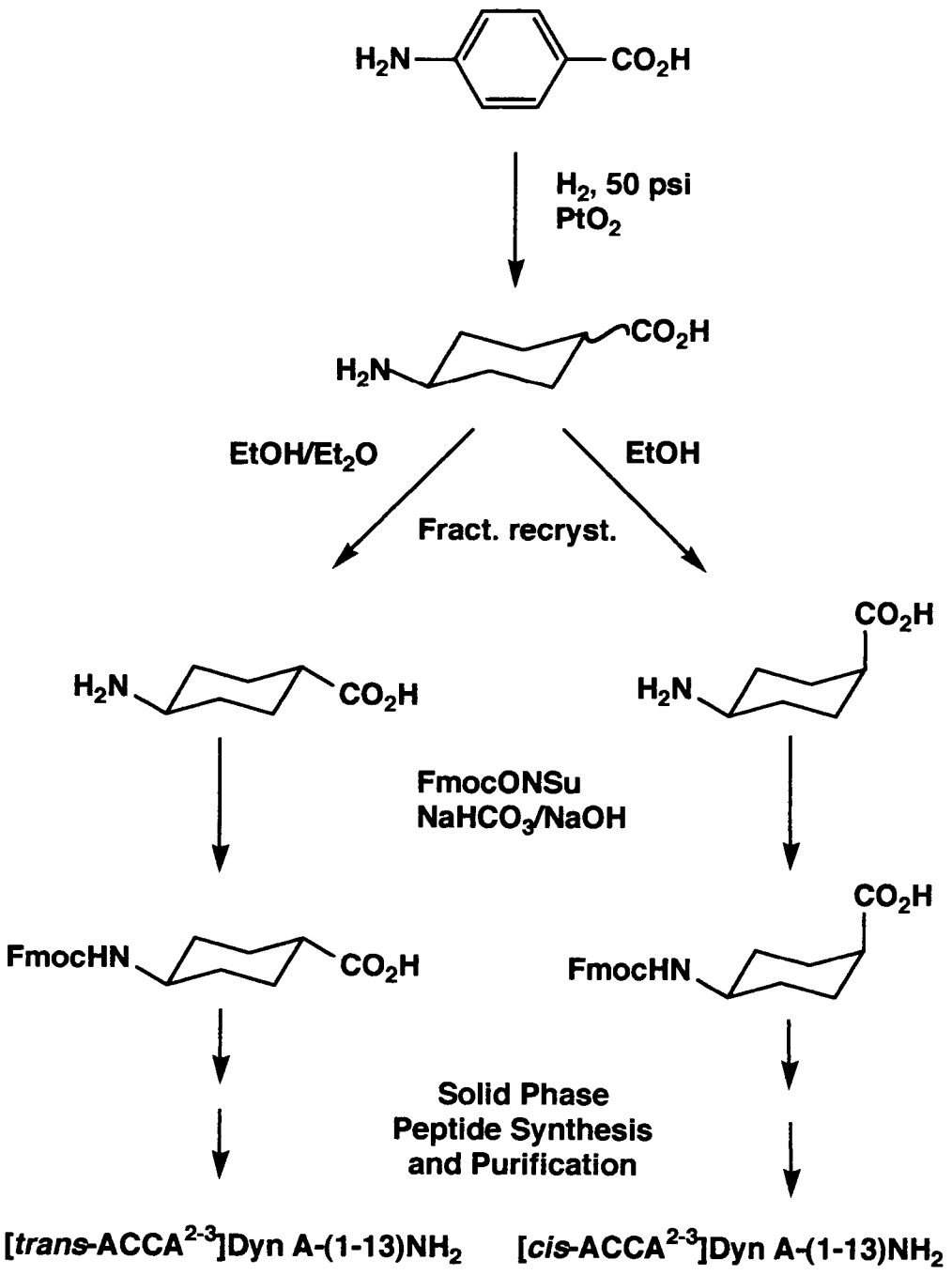


Fig. 7. ACCA Synthetic Scheme



recrystallization, each isomer was converted separately to its Fmoc (9-fluorenyl-methoxycarbonyl) derivative. Fmoc protection of these branched amino acids proved to be difficult, and literature procedures[74] had to be modified[75] to obtain the desired product (see Experimental Section). HPLC verified that Fmoc-*cis*-ACCA contained only the cis isomer. In the case of Fmoc-*trans*-ACCA, the contaminating cis isomer (R_t = 29.3 min) was not well resolved from the desired Fmoc-*trans*-ACCA (R_t = 29.7 min) by HPLC, so this mixture was used in the synthesis of the *trans*-ACCA analogue of Dyn A-(1-13)NH_2.

Both the cis and trans isomers were incorporated separately into Dyn A-(1-13)NH_2 (Fig. 7). The peptides were prepared as amides because of the enhanced metabolic stability of Dyn A-(1-13) amide vs. its corresponding acid.[59] The peptides were synthesized on a PAL resin[60] using Fmoc-protected amino acids by procedures described previously.[76] (See Chap I.) The peptides were deprotected and cleaved from the PAL resin using trifluoroacetic acid (TFA), and purified by preparative reverse phase HPLC. The purification of [*cis*-ACCA$^{2-3}$]Dyn A-(1-13)NH_2 was straightforward, while the purification of the trans analogue proved difficult due to the necessity of separating it from the contaminating cis analogue. This resulted in a very low yield of pure [*trans*-ACCA$^{2-3}$]Dyn A-(1-13)NH_2.

Opioid Receptor Binding Affinities and Opioid Activity. The peptides were evaluated for opioid receptor affinity at κ receptors by measuring the inhibition of binding of [^{3}H]bremazocine to guinea pig cerebellar membranes, and for μ and δ receptor affinities in rat forebrain membranes using[^{3}H]DAMGO ([D-Ala^2,MePhe^4,Glyol]enkephalin) and [^{3}H]DPDPE ([D-Pen^2,D-Pen^5]enkephalin), respectively (Table 6).

Table 6. Opioid Receptor Binding Affinities of Dyn A-(1-13)NH_2 and [ACCA]Dyn A-(1-13)NH_2 Analogues

	K_i (nM)			
Analogue	[^{3}H]Bremazocine (κ)	[^{3}H]DAMGO (μ)	[^{3}H]DPDPE (δ)	Ratio κ/μ/δ[1]
Dyn A-(1-13)NH_2	0.146 ± 0.004	0.194 ± 0.002	3.88 ± 0.09	1/1.3 / 27
[cis-ACCA$^{2-3}$]Dyn A-(1-13)NH_2	9.10 ± 0.92	117 ± 2	1910 ± 65	1/13 / 210
[trans-ACCA$^{2-3}$]Dyn A-(1-13)NH_2	13.4 ± 0.6	275 ± 2	1370 ± 53	1/ 21/ 103

[1] Ratio of $K_{i\kappa}/K_{i\mu}/K_{i\delta}$

Both [*cis*-ACCA$^{2-3}$]- and [*trans*-ACCA$^{2-3}$]Dyn A-(1-13)NH_2 bound to κ opioid receptors with modest affinity (K_i = 9-13 nM), with the *cis*-ACCA analogue exhibiting slightly greater affinity than the trans isomer. The affinity of these analogues for κ receptors is 1/60 to 1/90 that of the parent peptide Dyn A-(1-13)NH_2. Introduction of either isomer of ACCA into Dyn A-(1-13)NH_2 causes an even larger decrease in affinity for μ receptors, resulting in K_i's greater than 100 nM. Therefore, both of these analogues are κ selective, with [*trans*-ACCA$^{2-3}$]Dyn A-(1-13)NH_2 showing slightly better κ selectivity (κ/μ ratio = 1/21) than [*cis*-ACCA$^{2-3}$]Dyn A-(1-13)NH_2 (κ/μ ratio = 1/13). Both of these peptides had very little affinity for δ receptors (K_i's >1,000 nM).

Both the *cis*- and *trans*-ACCA Dyn A analogues show binding affinity similar to the Dyn A-(1-13)NH_2 analogues containing an L-amino acid at position 2 reported by Story, et al.,[66] all of which had K_i's in the 2-20 nM range. The ACCA substituted peptides, however, have better κ vs. μ selectivity than any of these 2-substituted dynorphin analogues. [Aib^2]Dyn A-(1-13),[77] which incorporates an α,α-disubstituted amino acid into position 2 similar to the disubstitution α to the amine of ACCA, surprisingly has lower affinity for κ receptors ($K_{i\kappa}$ = 50 nM). [Aib^2]Dyn A-(1-13) has 10- to 30-fold higher affinity for μ receptors ($K_{i\mu}$=10 nM) than [*cis*-] and [*trans*-ACCA$^{2-3}$]Dyn A-(1-13)NH_2; thus, the Aib^2-substituted peptide is μ selective while the ACCA-substituted peptides are κ selective.

[*cis*-ACCA$^{2-3}$]Dyn A-(1-13)NH_2 was evaluated for opioid activity in the guinea pig ileum (GPI) assay. Its potency (IC_{50} 4.09 μM, 95% confidence = 2.88-5.80 μM) was much lower than the parent Dyn A-(1-13)NH_2 (IC_{50} 0.24 nM, 95% confidence = 0.21-0.29 nM), but this analogue exhibited a full dose-response curve and naloxone antagonized its effects (data not shown.) The low GPI activity of [*cis*-ACCA$^{2-3}$]Dyn A-(1-13)NH_2 parallels the results obtained for the analogues containing L-amino acids in position 2.[66] There was insufficient trans compound to allow for its testing in the GPI.

Conclusions

[*Cis*-] and [*trans*-ACCA$^{2-3}$]Dyn A-(1-13)NH_2 are the first reported Dyn A analogues conformationally constrained in the "message" sequence that are selective for κ opioid receptors. The *cis*- and *trans*-ACCA$^{2-3}$-substituted peptides showed surprisingly similar κ, μ, and δ affinities when compared to each other and to Dyn A analogues containing an L-amino acid in position 2.[66] These results suggest that the Gly^2–Gly^3 peptide bond is not critical for κ opioid receptor affinity or selectivity. The similar binding affinities of the *cis*- and *trans*-ACCA Dyn A analogues, however, make it difficult to make conclusions concerning the bioactive conformation of the "message" sequence of Dyn A. The differences observed in receptor affinities and selectivity between the κ-selective [*cis*-ACCA$^{2-3}$]- and [*trans*-ACCA$^{2-3}$]Dyn A-(1-13)NH_2 and the μ-selective Dyn A analogue [Aib^2]Dyn A-(1-13)[77] could be due to differences in conformation, the larger size of ACCA vs. Aib, or the substitution at the position corresponding to the C_α of Gly^3 in the ACCA-substituted peptides.

The good discrimination of κ vs. μ receptors exhibited by the *cis*- and *trans*-ACCA derivatives of Dyn A-(1-13)NH_2 is encouraging and suggests that further modification at positions 2 and/or 3 might lead to Dyn A analogues with higher κ receptor affinity and opioid potency.

Experimental Section

Materials. The reagents and instrumentation used were those described previously.[76] (See Chap I.) Elemental analysis was performed by MWH Laboratories, Phoenix, AZ. NMR spectroscopy was done on a 400 MHz Bruker NMR in the Department of Chemistry at Oregon State University.

Hydrogenation of *p*-aminobenzoic acid to ACCA.[72] *p*-Aminobenzoic acid (Aldrich, 1.056 g, 7.71 mmol) in 30% EtOH (100 mL) was hydrogenated under 50 psi H_2, with PtO_2 (278 mg) as a catalyst, at room temperature overnight. This was repeated six times and the solutions combined. After removing the platinum by filtration, the filtrate was concentrated *in vacuo* until crystals appeared. The crystals were dissolved in H_2O and boiled, and the solution was treated with charcoal. ACCA was precipitated by the addition of 55 mL EtOH/ether (10/1), followed by additional ether until no more crystals appeared. This yielded 4.916 g (70.7%) of ACCA as a mixture of cis and trans isomers; TLC: R_f (C_{18}, reverse phase, MeOH/H_2O, 60/40) 0.33.

Separation of cis and trans isomers of ACCA.[73] The crystals were dissolved in water and EtOH was added to crystallize *cis*-ACCA. The crystals were collected and the process was repeated 3 times to obtain additional cis isomer. The remaining filtrate was concentrated and 55 mL EtOH/ether (10/1) added to crystallize the *trans*-ACCA . The crystals were collected and the process was repeated 3 times with the addition of excess ether. The isomers were then recrystallized from H_2O/EtOH (cis) or H_2O/EtOH/ether (trans) to yield 3.125 g (44.9%) of *cis*-ACCA and 1.259 g (18.1%) of *trans*-ACCA. Data for *cis*-ACCA follow: Mp: 258-264°C. FAB-MS: *m/z* 144 (M+1). ^{1}H NMR (d-TFA): δ 1.76-2.34 (m, 8H, -CH_2CH_2-), 2.85 (m, 1H, -C<u>H</u>CO_2H), 3.53 (m, 1H, -C<u>H</u>NH_2). Anal. ($C_7H_{13}NO_2 \cdot 0.4\ H_2O$): C, H, N. (See Appendix B.) HCl salt mp: 207-208°C (lit. mp: 217°C[73]). Data for *trans*-ACCA follow: Mp: 262-267°C. FAB-MS: *m/z* 144 (M+1). ^{1}H NMR (d-TFA): δ 1.60-1.70 (m, 4H, -CH_2-), 2.33 (m, 4H, -CH_2-), 2.52 (m, 1H, -C<u>H</u>CO_2H), 3.46 (m, 1H, -C<u>H</u>NH_2). Anal. ($C_7H_{13}NO_2 \cdot 0.4\ H_2O$): C, H, N. (See Appendix B.) HCl salt mp: 272-273°C (lit. mp: 273°C[73]).

Fmoc-*cis*- and Fmoc-*trans*-ACCA.[75] *Cis*-ACCA (393 mg, 2.73 mmol) and NaOH (109 mg, 2.73 mmol) were dissolved in H_2O (1.97 mL). A suspension of Fmoc-ON-Su (Bachem, 737 mg, 2.18 mmol) in THF (1.97 mL) was added to the ACCA solution, followed by additional H_2O and THF (1.97 mL of each). After 5 min $NaHCO_3$ (229 mg, 2.73 mmol) and THF (1.97 mL) were added; all reactants went into solution after 25 min. The reaction was stirred until TLC ($CHCl_3$/MeOH/AcOH, 89/10/1) indicated that all of the Fmoc-ON-Su had reacted (overnight). The solution was then acidified with excess 1.5 N HCl and the product, which precipitated out, dissolved in EtOAc. The HCl layer was extracted with additional EtOAc. The combined EtOAc extracts were then washed with 1.5 N HCl (2X), H_2O (2X), and saturated NaCl and dried over anhydrous $MgSO_4$ or Na_2SO_4. The solvent was evaporated *in vacuo* to give a thick oil. Dissolution in EtOAc, precipitation with hexane, and evaporation of solvent yielded 737 mg (92.6%) of Fmoc-*cis*-ACCA as a glassy solid. Mp: 128-130°C. FAB-MS: *m/z* 366 (M+1). HPLC[A] (0 to 75% B over 50 min, 1.5 mL/min): R_t = 29.3 min. ^1H NMR (d_6-DMSO): δ 1.40-1.58 (m, 6H, $-CH_2CH_2-$), 1.85 (m, 2H, $-CH_2-$), 2.38 (br s, 1H, $-C\underline{H}CO_2H$), 3.41 (m, 1H, $-C\underline{H}NH-$), 4.22 (m, 3H, Fmoc $-C\underline{H}C\underline{H_2}O-$), 7.29 (d, 1H, -NH-), 7.31 (t, 2H, Ar), 7.40 (t, 2H, Ar), 7.69 (d, 2H, Ar), 7.87 (d, 2H, Ar). Anal. ($C_{22}H_{23}NO_4$): C, H, N. (See Appendix B.)

Protection of the trans isomer (429 mg, 3 mmol) as described for *cis*-ACCA yielded 760 mg (86.7%) of Fmoc-*trans*-ACCA. FAB-MS: *m/z* 366 (M+1). HPLC[A] (0 to 75% B over 50 min, 1.5 mL/min): R_t = 29.7 min (Fmoc-*trans*-ACCA, 66%), R_t = 29.3 min (Fmoc-*cis*-ACCA, 33%). Data for an analytical sample recrystallized from THF/H_2O follow. Mp: 220-227°C. ^1H NMR (d_6-DMSO): δ 1.19-1.35 (m, 4H, $-CH_2CH_2-$), 1.85-1.94 (m, 4H, $-CH_2CH_2-$), 2.08 (br t, 1H, $-C\underline{H}CO_2H$), 3.18 (m, 1H, $-C\underline{H}NH-$), 4.20 (m, 1H, Fmoc $-C\underline{H}CH_2O-$), 4.26 (m, 2H, Fmoc $-CHC\underline{H_2}O-$), 7.22 (d,

1H, -NH-), 7.32 (t, 2H, Ar), 7.40 (t, 2H, Ar), 7.67 (d, 2H, Ar), 7.87 (d, 2H, Ar). Anal. ($C_{22}H_{23}NO_4$): C, H, N. (See Appendix B.)

Peptide Synthesis using Fmoc-protected Amino Acids. Both peptides were synthesized, cleaved from the resin, and purified by procedures described previously.[76] (See Chap I.) Before removing the Fmoc group from ACCA, the entire coupling sequence for that amino acid was repeated to ensure complete coupling. Following cleavage from the resin the peptides were purified by preparative reverse phase HPLC^B (0 to 50% B over 75 min, 20 mL/min for the cis analogue, and 15 to 45% B over 60 min, 20 mL/min for the trans analogue). Data for [*cis*-ACCA$^{2-3}$]Dyn A-(1-13)NH_2 follow. Yield: 30 mg (24%). FAB-MS: *m/z* 1614 (M+1). HPLC^A (0 to 75% B over 50 min, 1.5 mL/min): R_t = 19.5 min. Amino acid analysis: Tyr (1) 0.93, Phe (1) 0.99, Leu (2) 2.02, Arg (3) 3.15, Ile (1) 0.97, Pro (1) 0.97, Lys (2) 2.01. Data for [*trans*-ACCA$^{2-3}$]Dyn A-(1-13)NH_2 follow. Yield: 4.4 mg (3.3%). FAB-MS: *m/z* 1614 (M+1). HPLC^A (15 to 35% B over 40 min, 1.5 mL/min): R_t = 14.4 min. Amino acid analysis: Tyr (1) 0.79, Phe (1) 1.05, Leu (2) 2.02, Arg (3) 3.12, Ile (1) 0.99, Pro (1) 1.02, Lys (2) 2.02.

Binding and Guinea Pig Ileum Assays. Guinea pig cerebellar membranes and rat forebrain membranes were prepared and binding assays performed as previously described,[66] (Appendix A) except that 100 nM DAMGO was included in the incubation mixtures of [^{3}H]bremazocine with guinea pig cerebellar membranes (κ binding assays). Guinea pig ileum assays were performed as previously described.[66] (See Appendix A.) In experiments involving naloxone, the antagonist was added to the tissue bath 10 min prior to determination of the agonist dose-response curve.

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Notes

[A] Vydac 214TP54 column, solvent A = 0.1% TFA in H_2O, solvent B = 0.1% TFA in AcCN.

[B] Protein Plus preparative column, solvents A and B are as above.

Conclusion

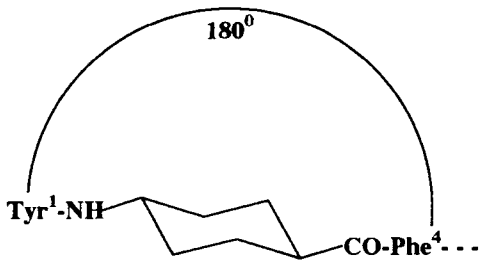
Designing an opioid drug with less abuse potential and fewer side-effects may still lie quite a distance in the future, but our understanding of the requirements that must be met grows each day. The findings in Chapters I and II may be small, but make significant contributions to this understanding. The study in Chap. I involved eliminating each of the positive charges in positions 6, 7, 9, 11, and 13 in Dyn A-(1-13)NH_2 by replacing each of the amino acids in these positions by Lys(Ac). The results indicated that Arg^9 is the only residue that could be replaced by a non-basic amino acid without losing κ-selectivity or potency. Therefore, this would be a good position through which to incorporate a conformational constraint in further studies of the "address" sequence of Dyn A. When the charge at positions 7, 11, or 13 is removed, all κ-selectivity is lost, so these positions are not options to alter further. Although removing the charge at position 6 lowers the opioid potency and makes [$Lys(Ac)^6$]Dyn A-(1-13)NH_2 nonselective for κ vs. μ receptors, it still retains high binding affinity. This means altering this position, with or without retention of the positive charge, could possibly lead to useful pharmacological tools in further studies.

Chapter II described the synthesis and pharmacological evaluation of the first reported analogues of Dyn A constrained in the "message" sequence that are selective for κ opioid receptors. When Chapter II was published, it still was not clear what could be concluded about the "message" sequence of Dyn A since the *cis*- and *trans*-ACCA-Dyn A analogues showed very similar receptor affinities. With further consideration several possibilities have become apparent. In light of the low affinity of these compounds, it is possible that an interaction between the peptide and receptor (for example a hydrophobic or van der Waals interaction with one of the aromatic rings) has been significantly disrupted in both analogues so that the remaining interactions are the same. Another

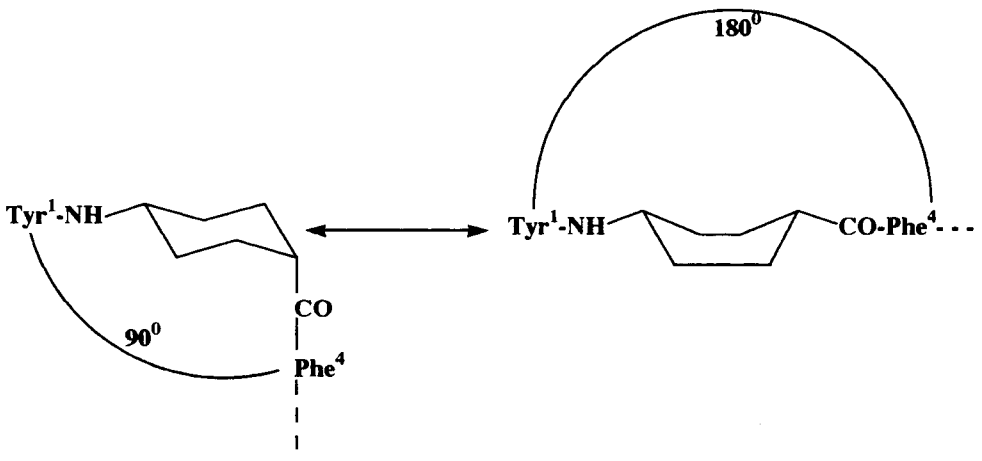
possible explanation of the similar affinities of [*cis*]- and [*trans*-ACCA$^{2-3}$]Dyn A-(1-13)NH_2 for opioid receptors is that, with free rotation around the bonds in residues 1 and 4, the aromatic rings of Tyr^1 and Phe^4 could have similar orientations at the receptor binding site regardless of whether *cis*- or *trans*-ACCA is in positions 2-3.

More importantly, in our initial analysis of the conformation of the "message" sequence of Dyn A, we assumed that the cyclohexane ring in ACCA would preferentially assume a chair conformation, as this is more thermodynamically stable than the boat conformation. In this case, the *trans*-ACCA analogue should have an angle of approximately 180° between Tyr^1 and Phe^4 (extended conformation; see Fig. 8), and the *cis*-ACCA analogue should have a 90° angle between Tyr^1 and Phe^4 (bent). However, if the cyclohexane ring assumes a boat conformation, in the *cis*-ACCA analogue the angle between Tyr^1 and Phe^4 becomes approximately 180° as in the *trans*-ACCA analogue. This could not only explain their similar binding data, but also suggests that the "message" sequence of Dyn A is in an extended conformation. Using the same reasoning and assuming the *trans*-ACCA ring is in a boat conformation, makes its Tyr^1 to Phe^4 angle 90°, but this possibility is even more thermodynamically unlikely. Thus, the bioactive conformation of the "message" sequence of Dyn A is probably not a bend or turn.

Fig. 8. Ring Conformations



trans-ACCA analogue



cis-ACCA analogue

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APPENDICES

Appendix A: Biological Assays

Binding Assays

Guinea Pig Brain Preparation and [^{3}H]Bremazocine Binding Assay. Frozen guinea pig brains obtained from Pel-Freez (Rogers, AR) were thawed on ice before the cerebellum was dissected away. The guinea pig cerebelli, weighing approximately 0.5 g, were suspended in 100 volumes ice-cold 20 mM HEPES, pH 7.4, by Polytron (Brinkman Instruments) for 15 seconds at setting 7.5. The homogenate was then centrifuged at 47,000 x g for 10 minutes at 4° C. The pellet was resuspended in buffer, preincubated for 45 minutes at 37° C and then recentrifuged. The final pellet was suspended in 100 volumes 20 mM HEPES, pH 7.4 and stored on ice until use in the binding assay.

The [^{3}H]bremazocine binding assays were carried out at 4° C for 3 hours. One mL of tissue was added to borosilicate glass tubes containing bestatin at a final concentration of 10 μM and 20 μL of peptide stock solution in increasing final concentrations of 0.01 to 10,000 nM in a final incubation volume of 2 mL. The assay was initiated with the addition of [^{3}H]bremazocine at a final concentration of 0.75 nM. Nonspecific binding was determined in the presence of 1 μM Dyn A-(1-13)NH_2. The binding reaction was terminated by rapid filtration over #32 glass filters (Scheicher & Schuell) using a Brandel M24-R Cell Harvester. The filters were presoaked for 2 hours in 0.5% polyethyleneimine to decrease nonspecific filter binding. The filter disks were then placed in minivials with 4 mL Cytocint (ICN Radiochemicals) and allowed to elute for 6 hours before counting in a Beckman LS6800 scintillation counter.

Rat Forebrain Preparation and [^{3}H]DAMGO and [^{3}H]DPDPE Assays. Frozen rat brains obtained from Pel-Freez (Rogers, AR) were thawed on ice before the cerebellum and brain stem were removed and discarded. The rat forebrains, weighing approximately 1.2 g, were suspended in 50 volumes ice-cold 50 mM Tris, pH 7.7, by Polytron for 15 seconds at setting 7.5. The homogenate was then centrifuged at 45,000 x g for 15 minutes at 4° C. The pellet was resuspended and recentrifuged in buffer as in the previous step. The second pellet was resuspended in buffer and preincubated for 45 minutes at 37° C, recentrifuged, and the final pellet was suspended in 80 volumes 50 mM Tris, pH 7.7 to yield a concentration of 0.7 - 1.0 mg protein/mL, as determined by the method of Lowry.[78]

The [^{3}H]DAMGO and [^{3}H]DPDPE binding assays were carried out at 4° C for 5 hours. One mL of tissue was added to borosilicate glass tubes containing bestatin, captopril, and L-leucyl-L-leucine at final concentrations of 10, 30, and 50 μM, respectively. A 20 μL aliquot of peptide stock solution was added in increasing final concentrations of 0.01 to 10,000 nM in a final incubation volume of 2 mL. The DAMGO binding reaction was initiated with the addition of 0.35 nM [^{3}H]DAMGO. Nonspecific binding was determined in the presence of 10 μM levorphanol. The reaction was terminated by rapid filtration over #32 glass filters (Scheicher & Schuell), which had been presoaked for 2 hours in 0.5% polyethyleneimine as described above, using a Brandel M24-R Cell Harvester. The DPDPE binding reaction was initiated with the addition of 2.5 nM [^{3}H]DPDPE. Nonspecific binding was determined in the presence of 10 μM unlabeled DPDPE and the reaction terminated by rapid filtration over Whatman GF/C fiber filters, presoaked as described above, using a Brandel M24-R Cell Harvester. The filter disks were then placed in minivials with 4 mL Cytocint, eluted for 6 hours, and counted in a Beckman LS6800 scintillation counter as described above.

Data Analysis. Competition data were fit by nonlinear regression analysis using the Public Procedure FITCOMP of the NIH Prophet Computer Resource, which employs an iterative process to minimize residual least squares. The IC_{50} values derived from competition analyses were converted to dissociation constants (K_i) using the Cheng and Prusoff equation;[69] the K_D values determined for tritiated bremazocine, DAMGO and DPDPE were 0.0549 nM, 0.314 nM and 7.63 nM, respectively.

Guinea Pig Ileum Assay. Guinea pigs weighing approximately 400 g were obtained from a commercial supplier (Simonsen, Gilroy, CA) and kept on a 12/12 h light/dark schedule with free access to food and water. Vitamin C supplementation was provided in water. Guinea pigs were killed by exposure to CO_2 and segments of the longitudinal muscle of the ileum were dissected free. Tissues were suspended in a 10 mL siliconized tissue bath in oxygenated Kreb's solution at 37° C. One gram of tension was applied to the tissue before beginning field stimulation (80 mV, 0.1 Hz, 0.5 sec duration). Peptidase inhibitors were initially investigated, but discontinued when no significant differences were observed with and without inhibitors for this series of dynorphin analogues.

Agonist activity was determined by observing inhibition of stimulated contractions with cumulative doses of dynorphin analogues added to the bath. Mean IC_{50} values were determined from 3 - 6 replications using tissues from different guinea pigs. Dyn A-(1-13)NH_2 IC_{50} values were determined as a control for each tissue following determination of the IC_{50} value for each test compound. Data from experiments in which tissues did not recover to at least 75% of maximal peak height after analogue treatment or experiments in which IC_{50} values for Dyn A-(1-13)NH_2 were abnormal were not used.

Appendix B: Analytical Data

Elemental Analysis of cis- and trans-ACCA

MW cis-ACCA · 0.4 H_2O = MW trans-ACCA · 0.4 H_2O = 150.3

ELEMENT	THEORETICAL %	% cis-ACCA	% trans-ACCA
C	55.8	55.9	55.9
H	9.2	9.3	8.8
N	9.3	9.7	9.3

Elemental Analysis of Fmoc-cis- and Fmoc-trans-ACCA

MW Fmoc-cis-ACCA = MW Fmoc-cis-ACCA = 365.0

ELEMENT	THEORETICAL %	% Fmoc-cis-ACCA	% Fmoc-trans-ACCA
C	72.3	72.4	72.1
H	6.4	6.4	6.3
N	3.8	3.8	3.8