DESIGN AND SYNTHESIS OF NOVEL LINEAR AND CYCLIC PEPTIDE LIGANDS FOR KAPPA OPIOID RECEPTORS

BY

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Table of Contents

Page

Page

Page

Page

Page

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List of Tables

Page

Page

Page

List of Figures

Page

Page

List of Schemes

Abbreviations

Abbreviations used for amino acids follow the rules of the IUPAC-IUB Joint Commission of Biochemical Nomenclature in *Eur. J. Biochem*. **1984**, *138*, 9-37. Amino Acids are in the L-configuration except where indicated otherwise. Additional abbreviations used in this dissertation are as follows:

5-TAMRA-SE: 5-carboxytetramethylrhodamine succinimidyl ester,

AC: adenylyl cyclase;

Alloc: allyloxycarbonyl;

ADP: adenosine 5'-diphosphate;

Atc: 2-aminotetralin-2-carboxylic acid;

Boc: *tert*-butyloxycarbonyl;

cAMP: cyclic adenosine monophosphate;

cDNA: complementary DNA;

Cha: cyclohexylalanine;

CHO: Chinese hamster ovary;

CNS: central nervous system;

CPM: cyclopropylmethyl;

CPP: cell penetrating peptide;

CSF: cerebrospinal fluid;

Dab: 2,4-diaminobutyric acid;

DADLE: [D-Ala^2,Leu^5]enkephalin;

DAMGO: [D-Ala^2,MePhe^4,glyol]enkephalin;

Dap: 2,3-diaminopropionic acid;

DBU: 1,8-diazabicyclo[5.4.0]undec-7-ene;

DCM: dichloromethane;

DIEA: *N*,*N*-diisopropylethylamine;

DMAP: 4-dimethylaminopyridine;

DMF: *N*,*N*-dimethylformamide;

DPDPE: *cyclo*[D-Pen^2,D-Pen^5]enkephalin;

DPLCE: [D-Pen^2, L-Cys^5]enkephalin;

Dyn A: dynorphin A;

ESI-MS: electrospray ionization mass spectrometry;

EL: extracellular loop;

E-2078: [N-MeTyr^1,N-MeArg^7,D-Leu^8]dynorphin A-(1-8)NHEt;

Fmoc: 9-fluorenylmethoxycarbonyl;

GDP: guanosine 5'-diphosphate;

GNTI: guanidinium naltrindole;

GPI: guinea pig ileum;

GPCR: G-protein coupled receptor;

GTP: guanosine-5'-triphosphate;

GTPγS: guanosine-5'-(3-thiotriphosphate);

HIV: human immunodeficiency viruses;

HOBt: 1-hydroxybenzotriazole;

HPLC: high-performance liquid chromatography;

i.c.v.: intracerebroventricular;

i.p.: intraperitoneal;

i.v.: intravenous;

IL: intracellular loop;

LH-RH: lutenizing hormone-releasing hormone;

Mtt: 4-methyltrityl;

MVD: mouse vas deferens;

Nal(1'): 1-naphthylalanine;

Nal(2'): 2-naphthylalanine;

Nbb: nitrobenzamidobenzyl;

Nle: norleucine;

norBNI: nor-binaltorphimine;

NMR: nuclear magnetic resonance;

NSAID: non-steroidal anti-inflammatory drug;

OAll: O-allyl;

*o*NBS: *ortho*-nitrobenzenesulfonyl;

ORL-1: opioid-receptor like-1;

PAL: peptide amide linker;

Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl;

PEG: poly(ethylene glycol);

Pip: 2-phenylisopropyl;

PNS: peripheral nervous system;

Ppa: phenylproprionic acid;

PS: polystyrene;

PyBOP: benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate;

RCM: ring-closing metathesis;

s.c.: subcutaneous;

SAR: structure-activity relationship;

SEM: standard error of mean;

SPPS: solid-phase peptide synthesis;

TAPA: H-Tyr-D-Arg-Phe-β-Ala-OH;

*t*Bu: *tert*-butyl;

TFA: trifluoroacetic acid;

Tic: 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid;

TIPS: triisopropylsilane;

TLC: thin layer chromatogram;

TM: transmembrane;

Tna: 1,2,3,4-tetrahydro-2-naphthoic acid;

Trt: trityl;

WHO: World Health Organization.

Abstract

Our research focuses on the development of potent and highly selective peptide ligands for kappa (κ) opioid receptors and examination of the structure-activity relationships (SAR) for activity at these receptors. Both κ opioid receptor agonists and antagonists have potential therapeutic applications for a variety of diseases. Dynorphin (Dyn) A is an endogenous heptadecapeptide agonist at κ opioid receptors. Dyn A-based peptide antagonists could be useful pharmacological tools for studying κ opioid receptors. Arodyn is an acetylated Dyn A analog identified in our laboratory that is a potent and highly selective κ opioid receptor antagonist. One main purpose of this dissertation research was to evaluate the role of the N-terminal 'message' sequence (especially Phe^1 and Phe^3) of arodyn in κ opioid receptor affinity, selectivity and efficacy. These two positions were substituted with other aromatic or nonaromatic residues. The other main purpose of this research was to develop conformationally restricted analogs of arodyn and Dyn A with high affinity and/or selectivity that can be used to explore the structural and conformational requirements for interaction of these ligands with κ opioid receptors, and to explore the SAR for agonist vs. antagonist activity at κ receptors. Different cyclization strategies were explored for the synthesis of cyclic arodyn and Dyn A analogs, including cyclization through lactam bond formation or ring-closing metathesis (RCM).

Chapter 1. Introduction

1.1 Background and significance

Since the discovery of opioid receptors, considerable research has been focused on understanding their physiological and pharmacological roles.[1] Opioid receptors belong to the superfamily of G-protein coupled receptors (GPCR).[2] Three different types of opioid receptors, mu (μ), kappa (κ), and delta (δ), have been identified and cloned.[1] Clinically used analgesic agents such as morphine are potent μ opioid receptor agonists with some serious side effects, such as respiratory depression, physiological and psychological dependence, and constipation.[1] During the last two decades, considerable effort has been focused on the development of κ selective opioid agonists, especially those acting on the peripheral nervous system, as potential analgesics without the significant clinical side effects associated with morphine and other μ receptor selective analgesic drugs.[3, 4] Further, κ receptor agonists have been shown to decrease the self-administration of cocaine, and this could lead to new approaches for the treatment of cocaine dependence for which there is currently no suitable therapy.[5-7] Kappa agonists can also be potentially used in the treatment of HIV-1 and HIV-1 related encephalopathy[8, 9] and as neuroprotective and anticonvulsant agents.[10] Kappa opioid receptor antagonists, on the other hand, may be useful for the treatment of opioid[11, 12] and cocaine[13, 14] addiction and as antidepressant agents.[15] Therefore, selective ligands for κ opioid receptors are very useful for studying the functions of κ opioid receptors at the molecular level, which in turn could be very important in the development of new therapeutic agents.

Our research focuses on the development of potent and highly selective peptide ligands, especially antagonists, for κ opioid receptors. Small molecule antagonists for κ

opioid receptors exhibit extremely long half-lifes *in vivo* (e.g. several weeks to more than one month), therefore limiting their use as pharmacological tools.[16-19] In contrast, peptide antagonists for κ opioid receptors are expected to have much shorter half-lifes *in vivo*. The peptide ligands are also proposed to have different binding modes compared with small molecules,[20-22] and therefore peptide ligands can be complimentary tools to small molecule ligands to study κ opioid receptors.

Dynorphin A (Dyn A, Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln), a heptadecapeptide first isolated from porcine pituitary, is proposed to be an endogenous ligand for κ opioid receptors.[23] Dyn A is a potent opioid peptide, but it is not very selective for κ opioid receptors and also exhibits significant affinity for μ and δ receptors. Dyn A has an identical N-terminal tetrapeptide sequence (Tyr-Gly-Gly-Phe) to most other mammalian opioid peptides while the C-terminal sequence is unique to Dyn A. Based on these features, Goldstein and co-workers proposed the "message-address" hypothesis for Dyn A (Figure 1.1).[24] According to this hypothesis the N-terminal "message" sequence is responsible for Dyn A's opioid receptor activity, while the C-terminal "address" sequence imparts affinity for κ receptors.

Figure 1.1: "Message-address" concept for Dyn A-(1-11) and arodyn

Dyn A-(1-11): Tyr-Gly-Gly-Phe-*Leu-Arg-Arg-Ile-Arg-Pro-Lys*

Arodyn: Ac-Phe-Phe-Phe-Arg-*Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-*NH_2

"Message" *"Address"*

Although a variety of peptide agonists (both linear and cyclic analogs) for κ-opioid receptors have been identified,[1, 25] until recently the search for peptide antagonists

has met with limited success. Compounds in which the N-terminus was converted to a tertiary amine with substitution of Tyr[1] with a tertiary amine on the α-amino group such as *N,N*-diallyl and *N,N*-diCPM (CPM = cyclopropylmethyl) derivatives are antagonists that also show reasonable affinity and selectivity for the κ receptors.[26-28] Dynantin, with the Tyr residue replaced with the des-amino tyrosine derivative, (2S)-Mdp ((2*S*)-2-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid), shows high affinity and is a κ selective antagonist.[29] [Pro[3]]Dyn A-(1-11)NH_2 is also identified as a highly selective antagonist for κ opioid receptors.[30]

To date there are only two conformationally constrained Dyn A analogs reported with antagonist activity at κ opioid receptors.[31, 32] Cyclodyn (*cyclo*^{N,5}[Trp[3],Trp[4],Glu[5]]Dyn A-(1-11)NH_2) is the first cyclic Dyn A analog with antagonist activity at κ opioid receptors.[31] Recently, a cyclic Dyn A analog cyclized in the C-terminal "address" domain, [N^{α}-benzyl-Tyr[1],*cyclo*(D-Asp[5],Dap[8])]Dyn A-(1-11)NH_2, was identified as a novel κ opioid receptor antagonist.[32] Synthesis of novel high affinity peptide κ opioid receptor antagonists with conformational constraints will aid in studying the interactions of the peptides with opioid receptors.

Chimeric Dyn A analogs have been designed as κ opioid receptor antagonists. Replacement of the N-terminal "message" sequence with a small μ opioid receptor antagonist followed by a construction of a combinatorial library resulted in the discovery of arodyn (Ac[Phe[1,2,3],Arg[4],D-Ala[8]]Dyn A(1-11)-NH_2), a potent and highly selective antagonist at κ receptors.[33] A primary goal of this dissertation is to study the effects of structural modifications of arodyn on κ opioid receptor affinity, selective, and efficacy.

As a potent κ opioid receptor antagonist, arodyn is also a promising lead compound for developing novel conformationally constrained κ opioid receptor antagonists.

1.2 Research projects and hypotheses

The main objective of this dissertation is to study the structural requirements for κ opioid receptor affinity, selectivity, potency, and efficacy, of arodyn as well as Dyn A(1-11)NH_2.

Arodyn is a potent and highly selective antagonist for κ opioid receptors. One primary goal is to identify arodyn analogs with higher potency. Both linear and cyclic arodyn analogs were designed and synthesized towards this goal. One analog previously prepared, [$NMePhe^1$]arodyn, shows higher affinity (2-fold) and selectivity (6-fold) for κ opioid receptors than arodyn.[34] During the synthesis of [$NMePhe^1$]arodyn, however, the arodyn (2-11) derivative was obtained as the major product.[35] Analysis indicated that Ac-NMePhe was lost from the completed peptide sequence during acidic cleavage of the peptide from the resin.[35] Because of the remarkable pharmacological activity of this compound, we studied the deletion reaction and synthesized stable analogs for the study of structure-activity relationships (SAR) (Chapters 3 and 4).

Alanine scan analysis suggested that Phe^1 and Phe^3 were two residues that contribute to arodyn's κ receptor affinity,[34] but initially it was unknown whether or not Phe was the optimal residue in these positions. Therefore, a small combinatorial library was designed previously where these two residues were substituted with other aromatic or non-aromatic residues (Tyr, Trp, 2'-naphthylalanine (Nal(2')), and cyclohexylalanine (Cha), etc.) to optimize these two residues.[35] To our surprise, several analogs exhibited

inverse agonist or agonist activity, although most of arodyn analogs synthesized were neutral antagonists for κ opioid receptors. The SAR for both agonist and inverse agonist activity were investigated further (Chapter 5).

As a linear peptide, arodyn can adopt numerous conformations. Therefore, the specific conformation responsible for receptor binding is unknown. Conformational constraint by cyclization is one approach that can be used to restrict the flexibility of peptide molecules, and therefore it is a valuable approach to study topographical requirements for receptor interactions.[25, 36, 37] In addition, cyclization of peptides may provide potent and selective ligands for receptors when appropriate conformational constraints are incorporated,[37] because a well-fit pre-organized conformation decreases the entropy penalty for receptor binding. Cyclic peptides are also often more stable to peptidases than linear peptides.[38-40] Arodyn itself shows a very short half-life of less than 2 minutes in rat brain homogenate.[40] Furthermore, cyclization may also increase membrane transport as well as metabolic stability as shown for some cyclic Dyn A analogs.[40]

We first synthesized cyclic arodyn analogs through cyclization between a free amine and a free carboxylic acid by amide bond formation. Cyclization was performed between the side chain of D/L-Asp and the side chain of Dap (2,3-diaminopropionic acid), Dab (2,4-diaminobutyric acid), Orn, or Lys. Base on the SAR of linear arodyn analogs, we chose to cyclize the peptide between the 2 and 5, 5 and 8, and 2 and 8 positions. We used different configurations of amino acids and/or positions for the cyclizations because different cyclizations can induce different conformations and

therefore could result in ligands with different affinity, selectivity and/or efficacy for κ opioid receptors (Chapter 6).

Ring-closing metathesis (RCM) has emerged as a very useful method of making cyclic organic compounds as well as cyclic peptides.[41-44] Compared with peptides cyclized by amide bond or disulfide bond formation, there are some advantages of using RCM for cyclization. The resultant carbon-carbon bond is more stable than an amide or disulfide bond. Furthermore, in contrast to cyclization via amide or disulfide bond formation, side chain functionalities can be maintained in peptides cyclized by RCM by using appropriate amino acid side chains. For example, the aliphatic side chain of Leu can be substituted with allylglycine (AllGly) for cyclization without changing its functionality. Phe^1 and Phe^3 are two important aromatic residues in arodyn for receptor binding.[34] By using allyl-protected Tyr for RCM, we can constrain these important residues while maintaining the aromatic functionality. Arodyn has three Phe residues in the N-terminus, and therefore serves as a good model peptide to study the scope and limitations of cyclization involving allyl-protected Tyr. Based on these assumptions, we designed several strategies of synthesizing cyclic arodyn analogs by RCM (Chapter 7).

Dyn A can also adopt numerous conformations, and the biologically active conformation at κ receptors is uncertain.[45-48] This inherent conformational flexibility may be one of the reasons that this peptide also exhibits significant affinity for μ and δ receptors, leading to low selectivity for κ receptors. To restrict the conformational mobility and enhance the metabolic stability, we also applied RCM for the synthesis of Dyn A analogs cyclized between the side chains of AllGly substituted in positions 2, 5 and 8 of this peptide (Chapter 8).

To be a potentially effective drug, a peptide is expected to be stable enough following administration to reach the biological target. In collaboration with Prof. Susan Lunte in the Department of Pharmaceutical Chemistry, we synthesized several Dyn A-(1-11)NH_2 analogs with different modifications such as N-terminal acetylation or alkylation, and cyclization. Several analogs exhibited significantly increased stability in plasma. Enhancement of transport across a blood-brain barrier (BBB) model was also observed. These pharmacokinetic studies will provide us fundamental knowledge of designing promising peptide κ opioid receptors ligands as therapeutic drugs.

E-2078 (([NMe-Tyr^1,NMe-Arg^7,D-Leu-NHEt^8]Dyn A(1-8)) is a stabilized Dyn A analog that shows comparable affinity but lower selectivity for κ receptor affinity compared to Dyn A.[49, 50] E-2078 has greatly increased stability towards peptidases because of several modifications.[49, 51] It produces analgesia following both intravenous (i.v.) and subcutaneous (s.c.) administration.[52, 53] This is the only Dyn A analog that has been through extensive pre-clinical and clinical evaluations. Therefore, this is an ideal compound for the evaluation of the effects of modifications on the transport of Dyn A. In order to synthesize the C-terminal ethylated amide Dyn A analog E-2078, we designed a convenient method of synthesizing C-terminal alkylated amide peptides on the PAL-PEG-PS resin, a commonly used resin for synthesis of C-terminal amide peptides (Appendix 1).

Dyn A, Dyn B and arodyn are similar to cell penetrating peptides (CPP) in possessing a high number of basic amino acid residues. In order to study the translocation of these peptides across the plasma membrane, we designed and synthesized fluorescent analogs of these peptides containing 5-carboxytetramethylrhodamine (5-TAMRA)[54-56]

attached to either the N- or C-terminus. From a synthetic point of view, attachment of a fluorescent tag to the N-terminus is easier to accomplish. However, the N-terminal residues of opioid peptides are critical for opioid receptor binding, and therefore the attachment of a bulky fluorescent group can affect the pharmacological activity. Therefore we also designed analogs where 5-TAMRA was attached at the C-terminus of these peptides. A polyethylene glycol-like linker was used to attach Lys(5-TAMRA) to Dyn A, Dyn B, and arodyn to minimize the influence of the bulky fluorescent tag on interactions with the κ opioid receptors (Appendix 2).

Arodyn is a potent and highly selective antagonist for κ opioid receptors that has very low affinity for μ opioid receptors. However, the study of the SAR shows that one analog, [CH_3OCO-NMePhe^1,Nal(2)^3]arodyn, exhibits much higher affinity at μ opioid receptors (K_i = 51.5 nM), even though it still exhibits a slight preference for κ opioid receptors (K_i ratio (κ/μ) = 1/2). We propose that at least three residues of the N-terminus of this peptide are required for the high affinity to μ opioid receptors. The "message-address" concept[24] suggests that the C-terminal "address" sequence of arodyn is important for affinity at κ opioid receptors. Therefore, deletion of the "address" sequence is expected to result in dramatically decreased affinity for κ opioid receptors. If the μ opioid receptor affinity can be preserved, we can switch the selectivity from κ to μ opioid receptors. The truncation of the C-terminal sequence also makes the peptides much smaller. These truncated peptides might represent a novel pharmacophore for μ opioid receptors. Several analogs truncated from the C-terminus of [CH_3OCO-NMePhe^1,Nal(2)^3]arodyn were designed and synthesized (Appendix 3). These peptides are currently undergoing pharmacological evaluations.

1.3 Conclusions

These research projects will be discussed in detail in subsequent chapters. The research described in this dissertation can enhance our understanding of the structural requirements for κ opioid receptor affinity, selectivity, potency, and efficacy. The conformationally constrained arodyn and Dyn A analogs synthesized by lactam bond or RCM in this research will advance our knowledge of how arodyn and Dyn A interact with κ opioid receptors. With the development of potent, stable peptide ligands and novel drug delivery for peptides, some of these analogs could lead to the development of therapeutic agents.

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Chapter 2. Literature Review

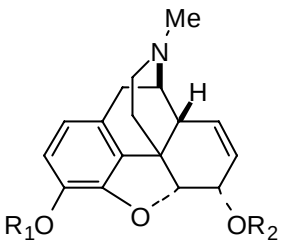
*Note that the compound numbers used in this chapter are only applicable within this specific chapter.

2.1 Opioid receptors

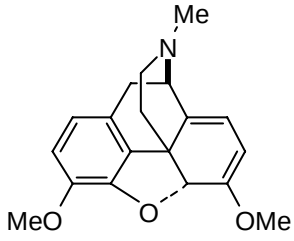
2.1.1 History

The interesting history of opioid receptors started over two thousand years ago. The word "opium" is derived from the Greek word "opos" meaning juice. Opium has been used as a medicinal agent for many centuries.[1, 2] In 1805, Sertürner isolated the major active ingredient in opium and named it morphine (**1**, Figure 2.1) after Morpheus, the Greek god of dreams.[3] Besides morphine, more than twenty other morphine-like alkaloids, such as codeine and thebaine (**2** and **3**, Figure 2.1), were identified from the latex of opium poppies.[4] Early structural modifications of morphine resulted in the discovery of some important compounds such as 3-ethylmorphine (**4**) and diacetylmorphine (heroin, **5**, Figure 2.1), which were used as medicines in the 19th century.[4] However, it was not until the mid-20th century that the pharmacological basis for these compounds was established. Continuous efforts to discover "ideal" analgesics with higher potency and fewer side effects led to the discovery of numerous new morphine derivatives and compounds derived from other sources.[5]

Figure 2.1: Structures of morphine-like compounds



Morphine, **1**: R_1=R_2=H
Codeine, **2**: R_1=Me, R_2=H
3-Ethylmorphine, **4**: R_1=Et, R_2=H
Heroine, **5**: R_1=R_2=Ac



Thebaine, **3**

Based on structure-activity relationship (SAR) studies, it was postulated for some time that these morphine-like alkaloids bind to specific receptor sites to cause their actions, and thus the concept of opioid receptors was proposed.[6] It was also reasoned that morphine and other synthetic analogs were not the endogenous ligands for the opioid receptors, and that some analgesic substance must exist within the brain to respond to environmental painful stimuli. However, it was not until the mid-1970s that the technology was developed to prove these two hypotheses.

2.1.2 Classification of opioid receptors

Multiple opioid receptors were postulated by Portoghese[7] and Martin[8] in the 1960s. The first classification of multiple types of opioid receptors came from the laboratory of Martin in 1976.[9, 10] Based on behavioral studies in chronic spinal dogs, Martin and his co-workers suggested the existence of three types of opioid receptors. These receptors were identified according to the prototype drugs used in the study: mu (μ) for morphine, kappa (κ) for ketocyclazocine and sigma (σ) for SKF 10047 or N-allylnormetazocine. The σ receptors were subsequently shown to not be opioid receptors because the opioid antagonist naloxone did not reverse agonist effects at these receptors.[11] In 1977, Kosterlitz and his co-workers proposed an additional type of opioid receptors, the delta (δ) receptors, based on studies with enkephalins in the mouse vas deferens (MVD).[12] More recently, a new opioid-like receptor was identified and cloned based on cDNA sequence homology with the other three known opioid receptors.[13] This new type of receptor does not bind the classical opioid agonists or antagonists with high affinity. Therefore this receptor was called the opioid-receptor like (ORL-1) receptor.

2.1.3 Endogenous opioid receptor ligands

In the mid-1970s, Hughes and co-workers were the first to isolate and purify two pentapeptides, Tyr-Gly-Gly-Phe-Leu (Leu-enkephalin, **6**, Table 2.1) and Tyr-Gly-Gly-Phe-Met (Met-enkephalin, **7**, Table 2.1), from mammalian brain tissue that have morphine-like activities.[14] These peptides were characterized *in vitro* as potent opioid agonists in the guinea pig ileum (GPI) and the MVD bioassays. These compounds were named "enkephalins" after the Greek word "kaphale" that means "from the head". Following the revolutionary discovery of the enkephalins, identification of other endogenous opioid peptides such as β-endorphin,[15] the dynorphins,[16] and the endomorphins,[17] were reported (Table 2.1).

Table 2.1: Endogenous opioid peptides

Peptide	Amino Acid Sequence	Selectivity[5]
Mammalian peptides		
Leu-enkephalin (**6**)	Y-G-G-F-L	δ>μ>>κ
Met-enkephalin (**7**)	Y-G-G-F-M	δ>μ>>κ
β-Endorphin (**8**)	Y-G-G-F-M-T-S-E-K-S-Q-T-P-L-V-T-L-F-K-N-A-I-I-K-N-Y-A-Y-K-G-E	μ≅δ
Dynorphin A (**9**)	Y-G-G-F-L-R-R-I-R-P-K-L-K-W-D-N-Q	κ>μ>δ
Dynorphin B (**10**)	Y-G-G-F-L-R-R-N-F-K-V-V-T	κ>μ>δ
α-Neoendorphin (**11**)	Y-G-G-F-L-R-K-Y-P-K	κ>δ,μ
β-Neoendorphin (**12**)	Y-G-G-F-L-R-K-Y-P	κ>δ,μ
Endomorphin-1 (**13**)	Y-P-W-F-NH_2	μ>>κ, δ
Endomorphin-2 (**14**)	Y-P-F-F-NH_2	μ>>κ, δ
Nociceptin/orphanin FQ (**15**)	F-G-G-F-T-G-A-R-K-S-A-R-K-L-A-N-Q	ORL1
Amphibian peptides		
Dermorphin (**16**)	Y-a[a]-F-G-Y-P-S-NH_2	μ>>δ>κ
Deltorphin (**17**)	Y-m-F-H-L-M-D-NH_2	δ>>μ>κ
[D-Ala^2]deltorphin I (**18**)	Y-a-F-D-V-V-G-NH_2	δ>>μ>κ
[D-Ala^2]deltorphin II (**19**)	Y-a-F-E-V-V-G-NH_2	δ>>μ>κ

[a]Small letter indicates D-configuration.

The endogenous mammalian opioid peptides are derived from their precursor proteins by proteases that recognize double basic amino acid residues just before and after the opioid peptide sequences.[18] There is a different precursor protein for each of the major mammalian opioid peptides. Proopiomelanocortin is the precursor of β-endorphin (**8**, Table 2.1) which has high affinity for both μ and δ opioid receptors. Proenkephalin A is similarly hydrolyzed to generate Leu- and Met-enkephalin (**6** and **7**) that have a slight preference for δ over μ opioid receptors. Prodynorphin is cleaved to produce multiple dynorphin peptides, namely dynorphin (Dyn) A and B (**9** and **10**, Table 2.1), and α- and β-neoendorphin (**11** and **12** respectively, Table 2.1). These opioid peptides have high

affinity for κ opioid receptors; however, they also have high affinity for other opioid receptors, resulting in only modest selectivity for κ opioid receptors. Most of the endogenous mammalian opioid peptides share a common N-terminal sequence (Tyr-Gly-Gly-Phe) but different C-terminal sequences. This observation led Goldstein and co-workers to propose the message-address hypothesis for dynorphin.[19] According to this hypothesis, the N-terminal (or "message") sequence is important for functional activity, whereas the unique C-terminal (or "address") sequence imparts affinity for κ receptor types.

In 1997, the endogenous opioid peptides for μ opioid receptors endomorphin-1 and -2 (**13** and **14** respectively, Table 2.1) were discovered. These two peptides show extremely high affinity and selectivity for the μ opioid receptors.[17] They have unique sequences where the second amino acid is a Pro instead of Gly as is found in most other mammalian opioid peptides. This substitution can restrict the conformational flexibility of the peptide backbone, and stabilize the conformation that binds to the μ receptors.

Shortly after the identification of the ORL-1 receptor, two research groups found a heptadecapeptide (**15**, Table 2.1) to be the endogenous peptide for this type of receptor.[20, 21] One of the research groups named this peptide nociceptin, as this peptide was reported to produce hyperalgesia.[20] The other research group named it orphanin FQ, because it was the ligand for an orphan receptor and the first and last amino acids in the peptide sequence are Phe (F) and Gln (Q), respectively.[21] The main difference between nociceptin/orphanin FQ and classical opioid peptides is that the first amino acid of this peptide is Phe instead of Tyr as found in other mammalian opioid peptides. This partially explains this peptide's high selectivity for ORL-1 over classical opioid receptors.

Peptides with opioid activity have also been isolated from sources other than mammalian tissue (Table 2.1). Dermorphin (**16**), a μ receptor selective peptide isolated from the skin of South American frogs, is about 100 times more potent than morphine in *in vitro* assays.[22] Later in 1989, the potent and highly δ opioid receptor selective peptide agonists deltorphin, [D-Ala^2]deltorphin I, and [D-Ala^2]deltorphin II (**17**-**19** respectively, Table 2.1) were also isolated from frog skin.[23-25] The unique feature of these amphibian opioid peptides is that the second amino acid has the D-configuration. DNA only encodes L-amino acids, so the D-amino acid arises from a post-translational modification.[26] Another major difference from mammalian opioid peptides is that the important aromatic residues Tyr and Phe in the amphibian peptides are separated by only one amino acid.

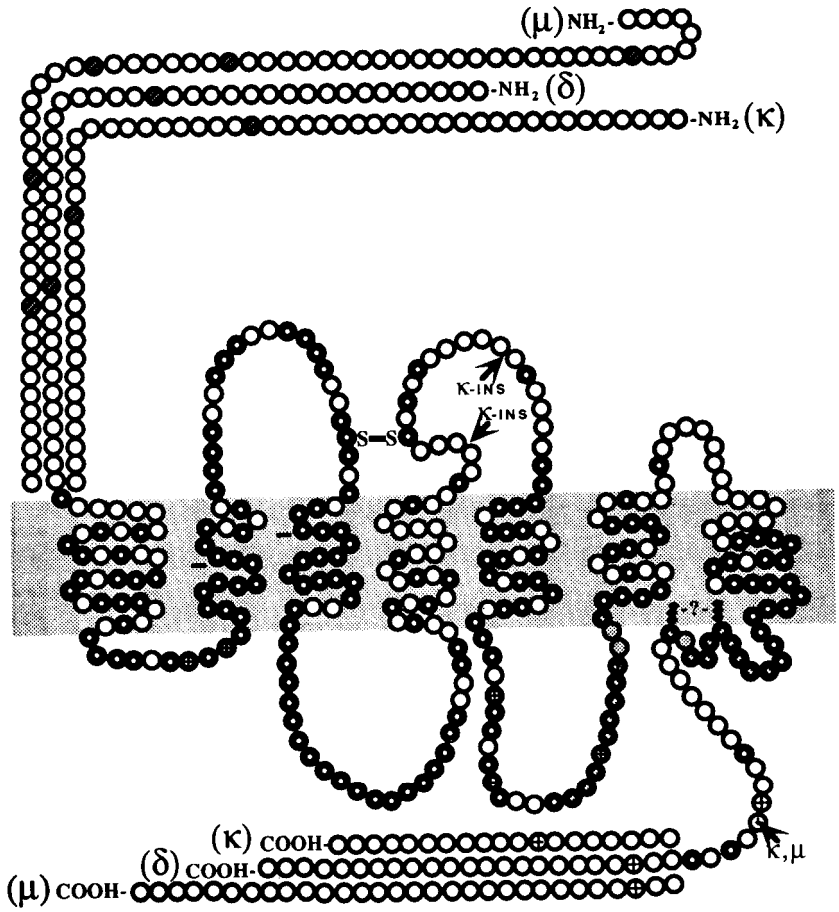
The discovery of these endogenous opioid peptides also promoted the use of the term "opioid". The term "opiate" was used extensively until the 1980s to describe any alkaloids extracted from poppy pods and their semi-synthetic and synthetic counterparts. The discovery of endogenous peptides that had pharmacological actions similar to morphine prompted a change in nomenclature. In terms of structure, these peptides are not directly related to morphine, but their actions are similar to these produced by morphine. At that time, the term "opioid" was introduced, meaning opiate- or morphine-like in terms of pharmacological actions. A broad group of opium alkaloids, synthetic derivatives related to opium alkaloids, and the many naturally occurring and synthetic peptides with morphine-like pharmacological activities are now all called opioids. In addition to having pharmacological actions similar to morphine, an opioid agonist must be antagonized by an opioid antagonist such as naloxone to be classified as an opioid.

2.1.4 Molecular pharmacology of opioid receptors

Opioid receptors belong to the rhodopsin subclass within the superfamily of G protein-coupled receptors (GPCR) (Figure 2.2), which are characterized by the presence of seven hydrophobic transmembrane (TM) helices, an extracellular N-terminus and three extracellular (EC) loops, as well as an intracellular C-terminus and three intracellular (IC) loops.[27] The three major types of opioid recepors, μ, δ, and κ, were cloned and fully characterized in the early 1990s. Delta opioid receptors from mouse were the first cloned opioid receptors, reported by Kieffer et al.[28] and Evans et al.[29] Yusuda et al. subsequently reported the cloning of both mouse κ and δ receptors.[30] Later on, cloning of these three opioid receptor types from a variety of species, including human beings, were reported.[31-33] These receptor proteins have very similar size and are highly conserved, especially in the TM and IC domains (Figure 2.2). All three opioid receptors have almost identical sequences in the second and third IC loops (Figure 2.2). This explains their overlapping physiological functions. Sequencing of the cDNA insert revealed that there are 372 amino acids for mouse and rat δ opioid receptors, 380 amino acids for κ opioid receptors, and 398 amino acids for rat μ opioid receptors. There is 97% sequence identity between rat and mouse δ opioid receptors, suggesting a high degree of homology among species.[34] There is about 61% amino acid sequence identity between rat μ and δ opioid receptors and 61% amino acid sequence identity between mouse κ and δ opioid receptors.[30] The deduced amino acid sequences of opioid receptors revealed the presence of two cysteine residues in the second and third EC loops that are linked by a disulfide bridge. This disulfide bridge appears to be important for the structural stability of the ligand-binding pocket of GPCRs.[35] A conserved aspartate residue in TM III is thought to serve as the

counter-ion for the positive charge which is present in most alkaloid and peptidic opioids.[36]

Figure 2.2: Structural homology of the μ, κ, and δ opioid receptors[36]



2.1.5 Signal transduction mechanisms of opioid receptors

Opioid receptors are coupled to the G_i/G_o family of G-proteins at their IC domain. G-proteins are heterotrimers, composed of three subunits, α, β, and γ.[27] Following binding of an agonist, the opioid receptors undergo a conformational change, which promotes the release of guanosine 5'-diphosphate (GDP) from the α subunit, followed by binding of guanosine 5'-triphosphate (GTP) to the same location. This results in the dissociation of the G-protein from the opioid receptor and the dissociation of the GTP-bound α subunit from the βγ dimer. Then both the GTP-bound α subunit and the βγ dimer can associate with various effector molecules to modulate biological functions.[37, 38] This activation is terminated when the GTP bound to the α subunit is hydrolyzed to GDP, leading to the re-association with the βγ subunits to reform the G-protein heterotrimer. There are many different isoforms of α, β, and γ subunits. α_i or α_o isoforms of the α subunit couple with opioid receptors. Activation of the opioid receptors can inhibit adenylyl cyclase (AC) activity, which results in decreased production of cyclic adenosine monophosphate (cAMP). The reduction in cAMP formation decreases the level of neurotransmitter release by reducing the activity of cAMP-dependent protein kinases. These coordinated events can produce analgesia.[39]

Opioid receptors can also be coupled to ion channels through G-proteins.[40, 41] Activation of opioid receptors can result in inactivation of voltage-dependent Ca^{++} channels, which leads to reduced neurotransmitter release. Activation of opioid receptors can also result in activation of K^+ channels. This induces membrane hyperpolarization, the most important action component of postsynaptic inhibition.

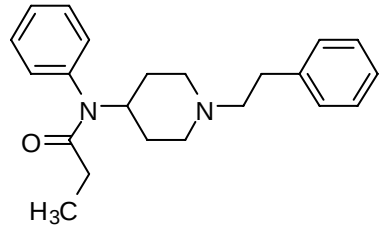
2.1.6 Clinical applications and side effects of opioids

Opioid receptors and their ligands are involved in a variety of pharmacological functions.[5] The endogenous opioid system is critical in maintaining the pain inhibitory system of the human body. Opioids, especially μ opioid receptor agonists, are used for the treatment of moderate to severe pain both acutely and chronically.[42] According to the World Health Organization (WHO)'s proposal for pain control, the selection of compounds should follow a three-step analgesics ladder in which compounds with increasing potency and efficacy are used.[43] The first step starts with a single use of a non-opioid analgesic such as a non-steroidal anti-inflammatory drug (NSAID). If the pain control is not sufficient, a weak opioid (such as codeine) may be added. If a further increase in analgesic efficacy is needed, such as for late-stage cancer pain, the weak opioid should be replaced by a strong opioid, e.g. morphine.

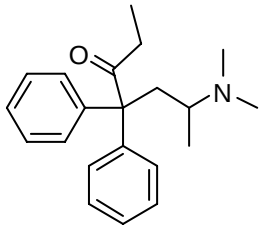
Morphine (**1**, Figure 2.1) is still the most widely used narcotic analgesic drug for severe pain and is regarded as the gold standard for pain treatment.[44] Structural modifications of morphine resulted in many agents with higher potency and/or less side effects. Codeine (**2**, Figure 2.1) is a component of opium extract. Compared with morphine, the analgesic potency of codeine is 5-10 fold lower. It is mainly used for the treatment of mild to moderate pain and for cough inhibition.[45] Fentanyl (**20**, Figure 2.3), which is about 80 times more potent than morphine, has been used as an adjunct in anesthesia.[5] A major advantage of fentanyl over morphine for anesthetic procedures is its shorter duration of action (1-2 h). Methadone (**21**, Figure 2.3) is a synthetic agent that is used mainly as a maintenance agent for the treatment of opioid addiction. These agents are agonists at the μ opioid receptors. In contrast, mixed agonists/antagonists such as

butorphanol, buprenorphine and pentazocine (**22-24**, respectively, Figure 2.3) generally interact with two different (μ and κ) opioid receptors to provide analgesic activity while exhibiting reduced potential for side effects that are mainly mediated by μ opioid receptors.[5] However, their clinical application is limited because the analgesic effects are limited compared to ligands acting solely as μ opioid receptor agonists. Furthermore, some mixed agonists/antagonists are related with dysphoria, typical side effects of κ opioid receptor agonists.[46, 47] Mu opioid receptor antagonists such as naloxone and naltrexone (**25** and **26** respectively, Figure 2.3), on the other hand, are used clinically to reverse the effects of an opioid overdose.[5]

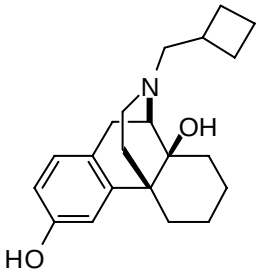
Figure 2.3: Some representative drugs acting on opioid receptors.



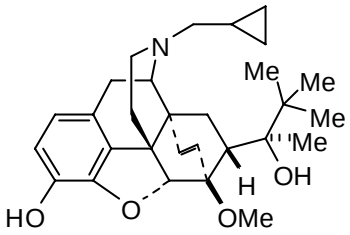
Fentanyl, **20**



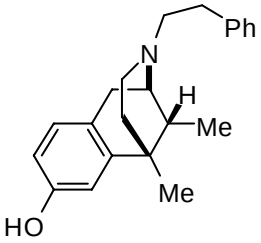
Methadone, **21**



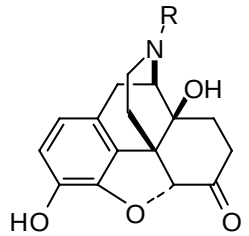
Butorphanol, **22**



Buprenorphine, **23**



Pentazocine, **24**



Naloxone, **25**: R=allyl
Naltrexone, **26**: R=cyclopropylmethyl

Some severe side effects are associated with the use of μ opioid agonists. They can induce respiratory depression via inhibition of the respiratory center of the medulla

oblongata, causing a decreased response to the CO_2 level of the blood.[48] This is the major cause of death resulting from an opioid overdose. Mu opioid agonists induce a feeling of well-being or euphoria, which is mediated by the release of dopamine within the limbic system.[49] Repeated activation of the μ opioid system may induce dependence, which leads to addiction and compulsive drug-seeking behavior. Patients treated with long-term opioid therapy develop tolerance, requiring a higher dose to achieve the same response.[5] Mu opioid agonists also inhibit gastrointestinal motility and fluid secretion. The reduced motility and fluid secretion can lead to constipation, which is the most common side effect of chronic opioid treatment.[50]

These side effects are mediated via μ opioid receptors, and extensive research on the development of synthetic opioids without addiction or dependence liability has met with limited success.[2] There has been considerable interest in ligands for other opioid receptors as potential analgesic agents with decreased side effects. For example, δ agonists may exhibit antinociceptive effects with lower dependence liability and respiratory depression than μ receptor agonists.[51] Delta opioid agonists, however, do not produce as strong analgesic effects as do μ opioid agonists.[52] Furthermore, some δ receptor agonists cause seizure.[53] Kappa opioid agonists have been shown to be analgesics without the clinical side effects caused by μ agonists;[54] however, they have been reported to cause other side effects such as dysphoria.[46, 47] Recently, some κ opioid receptor agonists were reported to have the potential to be used as peripheral analgesics without the central nervous system (CNS) side effects.[55]

2.1.7 *In vitro* evaluation of opioids

Following the synthesis of opioid ligands, they are evaluated for their pharmacological activities *in vitro*. Radioligand binding assays (to determine receptor affinity and selectivity) and functional assays (to determine efficacy and potency) are used to characterize opioid ligands. [^{3}H]DAMGO ([D-Ala^2,MePhe^4,glyol]enkephalin), [^{3}H]DPDPE (*cyclo*[D-Pen^2,D-Pen^5]enkephalin), and [^{3}H]U69593 (**27**, Figure 2.4) are most often used for radioligand binding assays for μ, δ, and κ receptors, respectively.[5] Chinese hamster ovary (CHO) cells and other cell lines expressing one type of opioid receptors have been used extensively in radioligand binding assays.

The intrinsic activities of opioid ligands are generally measured in functional assays using either isolated tissue preparations or cell lines expressing cloned receptors.[5] Tissue preparations such as the electrically stimulated GPI and MVD have been used most extensively for the evaluation of the activity of opioid ligands. In these assays, the primary effect of opioid agonists is the inhibition of electrically stimulated smooth muscle contraction. However, the activity of compounds in these assays can be complex, as the tissues contain more than one opioid receptor type. The GPI contains predominantly μ and κ opioid receptors, whereas the MVD contains all three types of opioid receptors. With the cloning of the opioid receptors, cell-based assays have largely replaced assays using isolated tissue preparations. Cell-based assays measuring either AC activity or [^{35}S]GTPγS binding are commonly used to determine the efficacy and potency of opioid ligands. As discussed in Section 2.1.5, activation of opioid receptors results in the inhibition of AC activity and reduction in the production of cAMP; this can be used as a functional assay to evaluate the efficacy and potency of opioid ligands.[56] GPCR

activation results in an increase in [^{35}S]GTPγS binding, and therefore the binding of [^{35}S]GTPγS to cell membranes expressing opioid receptors can be used to measure the efficacy and potency of an opioid compound.[57-59]

2.2 Kappa opioid receptors

2.2.1 Significance of kappa opioid receptors

Activation of κ opioid receptors can produce antinociceptive effects in a variety of experimental pain models.[5] Unlike μ agonists, κ agonists lack the side effects of respiratory depression, addiction, and constipation. Therefore, they were initially viewed as an attractive alternative to μ agonists and safer analgesics. However, the effectiveness of κ opioid agonists depends on the pain type. For some types of pain (e.g. thermal) κ opioid agonists are not as effective as agents acting on μ receptors.[5] Furthermore, centrally acting κ opioid agonists can produce side effects such as dysphoria,[46, 47] limiting their clinical use. Recently, studies have established that κ opioid agonists can produce analgesic peripherally both in somatic and visceral pain models, particularly in certain conditions involving inflammation.[60] Peripherally acting κ receptor agonists do not have the CNS side effects such as dysphoria, and therefore there has been an interest in developing peripheral κ agonists.[5]

Activation of κ opioid receptors opposes a variety of μ opioid receptor-mediated actions in the brain and spinal cord.[61] Compared to μ and δ opioid agonists, which increase dopamine levels in the nucleus accumbens, activation of κ receptors results in decreased dopamine levels in the nucleus accumbens.[62] Cocaine blocks the re-uptake of dopamine, which leads to increased dopamine in the extracellular space. Kappa receptor

agonists can decrease dopamine levels, and therefore can act as functional antagonists of cocaine.[63]

Kappa receptor agonists, especially the arylacetamide series of agonists, have been shown to have anticonvulsant and neuroprotective properties, and therefore have the potential for the treatment of epilepsy, stroke, or trauma resulting from brain or spinal cord injury.[64] Kappa agonists have also been shown to cause the down-regulation of HIV expression in microglia[65] and CD4+ lymphocytes.[66]

Kappa receptor antagonists can be used as valuable tools for the study of the structural and functional characteristics of κ opioid receptors. Currently there is also increased interest in the development of κ antagonists as clinical agents. Kappa opioid receptor antagonists have been shown to have antidepressant activity in the forced swim test in rats.[67] Prevention of activation of κ opioid receptors can facilitate the long-term maintenance of cocaine-abstinent patients. Two structurally unrelated κ receptor antagonists both block stress-induced reinstatement of cocaine seeking behavior[68, 69] and therefore κ receptor antagonists have the potential to be used therapeutically in the treatment of cocaine addiction. Kappa opioid receptor antagonists can decrease the withdrawal signs in opioid addicted subjects, and therefore also have potential application in the treatment of opioid addiction.[70, 71]

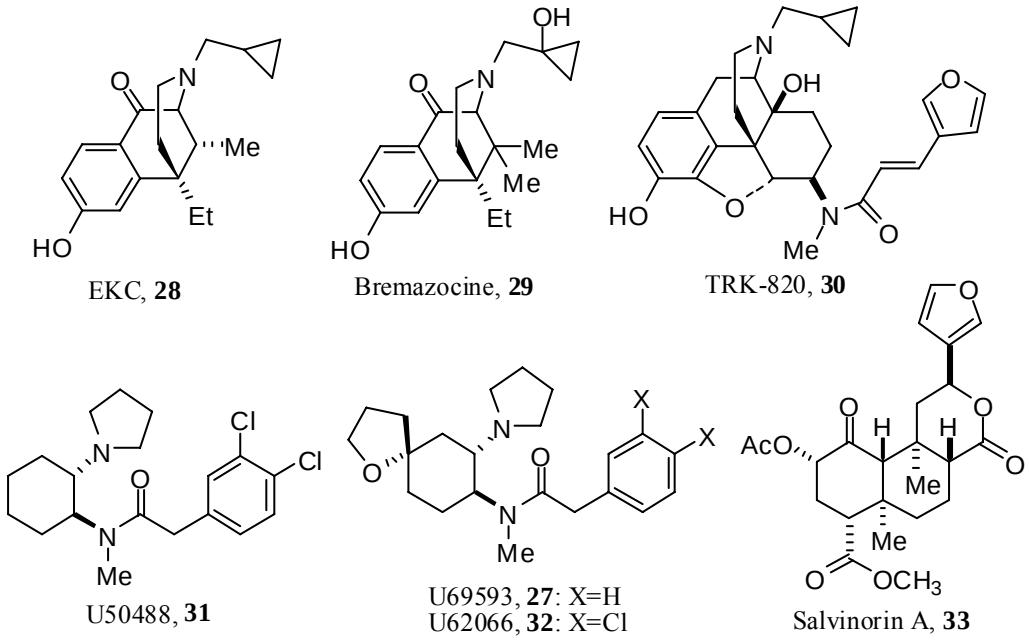
2.2.2 Kappa receptor selective ligands

2.2.2.1 Agonists

Unlike μ and δ opioid receptors, the identification of potent and highly selective κ receptors ligands was initially limited. Early κ opioid receptor agonists identified were

benzomorphan derivatives such as ethylketocyclazocine (EKC, **28**)[10, 72] and bremazocine (**29**) (Figure 2.4).[73] However, these compounds exhibit low selectivity for κ over other opioid receptors. The morphinan derivative TRK-820 (**30**, Figure 2.4) exhibits high potency and moderate κ opioid receptor selectivity over μ and δ opioid receptors. This κ receptor selective agonist is 85-fold more potent as an analgesic than morphine.[74] The discovery of the highly κ receptor selective arylacetamides derivatives resulted in significant advances in κ receptor pharmacology. U50488 (**31**, Figure 2.4) has 50-fold selectivity for κ over μ opioid receptors.[75] Introduction of a spiro ether group on the cyclohexane ring resulted in U69593 (**27**) and U62066 (**32**, Figure 2.4), both of which show high affinity and selectivity for κ over μ and δ opioid receptors.[76, 77] Recently, the natural product salvinorin A (**33**, Figure 2.4), the main active ingredient of *Salvia divinorum* extracts that have been used in the USA as a legal hallucinogen, was identified as a highly potent and selective κ opioid receptor agonist.[78] Salvinorin A is also the first naturally occurring non-nitrogenous opioid receptor agonist.

Figure 2.4: Structures of some nonpeptide κ opioid receptor agonists



Endogenous peptides Dyn A and B (**9** and **10**, Table 2.1) have high affinity for κ opioid receptors with only modest selectivity over μ and δ opioid receptors.[79] Further modification has led to the identification of a variety of Dyn A analogs with improved κ receptor affinity, selectivity, and/or potency (see Section 2.3.2 below).

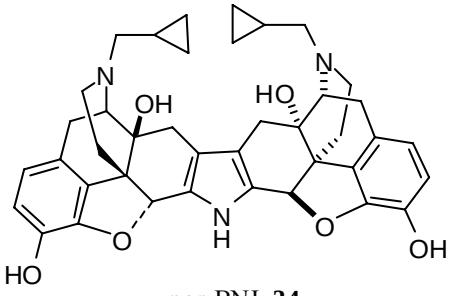
Kappa opioid receptor agonists have also been discovered by the application of combinatorial chemistry. A tetrapeptide κ receptor agonist, D-Phe-D-Phe-D-Nle-D-Arg-NH_2 (Nle = norleucine), shows high activity, κ receptor selectivity, and peripheral selectivity.[80]

2.2.2.2 Antagonists

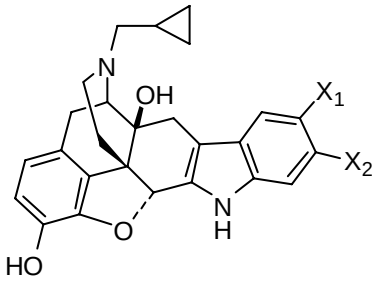
A number of κ opioid receptor antagonists have been discovered during the past two decades (Figure 2.5). The bivalent ligand nor-binaltorphimine (nor-BNI, **34**, Figure

2.5) was identified as a highly potent and selective κ opioid antagonist.[81] Extensive SAR studies of the δ opioid receptor antagonist naltrindole resulted in the identification of 5'-guanidinium naltrindole (5'-GNTI, **35**, Figure 2.5) as a κ selective antagonist.[82] Interestingly, the 6'-guanidinium naltrindole (6'-GNTI, **36**, Figure 2.5) is a potent κ opioid receptor selective agonist.[83] JDTic (**37**, Figure 2.5) is the first phenylpiperidine derived compound that is a potent and selective antagonist for κ opioid receptors.[84] It shows much higher κ opioid receptors affinity (K_i = 0.02 nM) and selectivity (570 and >16,000 fold selective over μ and δ opioid receptors, respectively) than other ligands.

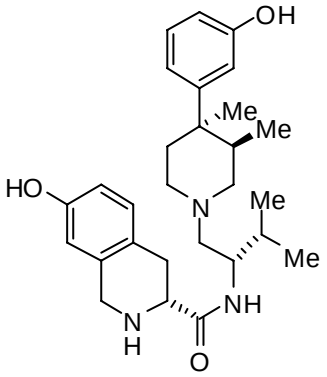
Figure 2.5: Structures of some κ opioid receptor antagonists



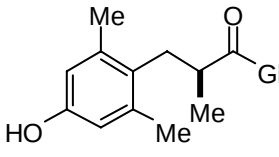
nor-BNI, **34**



5'-GNTI, **35**: X_1=NHC(NH)NH_2, X_2=H (Antagonist)
6'-GNTI, **36**: X_1=H, X_2=NHC(NH)NH_2 (Agonist)



JDTic, **37**



Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-NH_2

dynantin, **38**

Ac-Phe-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2

arodyn, **39**

However, all three antagonists exhibit extremely long half-lifes *in vivo* (ranging from several days to several weeks),[85-88] thus limiting their usage as pharmacological tools. Therefore, it is highly desirable to identify κ receptor antagonists with shorter duration of activity. Because of their intrinsic lability to peptidase metabolism, peptide ligands usually show shorter half-lifes *in vivo*.

Modifications of Dyn A have resulted in analogs with antagonist activity. According to the "message-address" concept, the N-terminal "message" sequence of Dyn A is responsible for opioid receptor activation.[19] Therefore, modifications to the N-terminal sequence were proposed to convert Dyn A analogs to antagonists. Dynantin (**38**, Figure 2.5), with replacement of the Tyr residue with a des-amino tyrosine analog, is an antagonist with high affinity and κ receptor selectivity.[89] Arodyn (Ac[$Phe^{1,2,3}$,Arg^{4},D-Ala^{8}]Dyn A(1-11)NH_2, **39**, Figure 2.5), an N-acetylated Dyn A(1-11)-NH_2 analog, is also a potent and highly selective antagonist at κ opioid receptors.[90] More details on Dyn A analogs with antagonist activity will be discussed in Section 2.3.4 of this chapter.

The recently discovered CJ-15,208 (*cyclo*(-D-Pro-Phe-(L/D)Trp-Phe-)), isolated from *Ctenomyces serratus* ATCC15502 fungus, is the first reported cyclic tetrapeptide that has been reported as an antagonist with modest selectivity for κ over μ opioid receptors.[91]

2.3 Dynorphin A

2.3.1 Pharmacological activities of Dyn A

Dyn A (**9**, Table 2.1) is the best studied endogenous ligand for κ opioid receptors. It was first isolated from porcine pituitary gland[92] and is widely distributed in the central

nervous system (hypothalamus, striatum, spinal cord, etc.) and in peripheral organs.[93] Dyn A-(1-13) has been shown to have potent opioid activity in several assays.[92] It inhibits the electrically stimulated twitch of the GPI longitudinal muscle. This peptide is extraordinarily potent, about 700 and 50 times more potent than [Leu]enkephalin and β-endorphin (**6** and **8**, Table 2.1), respectively, in the GPI. Because of its extraordinary potency, this peptide was named 'dynorphin' from the Greek word *dynamis* meaning strength or power. Its unusually high potency is believed to result from the C-terminal sequence (the "address" sequence,[19] see below) that imparts κ opioid receptors affinity.

Dyn A is implicated in motor dysfunction, cardiovascular effects, inflammatory response, feeding behaviors, and immunomodulation, as well as a variety of other biological functions.[94, 95] Some of the side effects such as motor dysfunction are mediated through non-opioid mechanisms.[96-98] Dyn A (1-13) has been shown to suppress opiate withdrawal and tolerance when administered intravenously to morphine-dependent mice.[99] Dyn A (1-13) has also been shown to modulate both acute and chronic opioid effects, and has direct analgesic effect in the writhing assay.[100, 101]

2.3.2 Structure-activity relationships of Dyn A

Since the discovery of Dyn A as an endogenous ligand for κ opioid receptors, the SAR of this peptide has been explored.[5, 102, 103] Dyn A has the N-terminal sequence Tyr-Gly-Gly-Phe, which is the same as most endogenous mammalian opioid peptides; however, the C-terminal sequence of this peptide is different from other opioid peptides. Based on these features, Goldstein and co-workers proposed the "message-address" hypothesis for Dyn A (Figure 1.1).[19] According to this hypothesis the N-terminal

"message" sequence is responsible for Dyn A's opioid receptor affinity and activity, while the C-terminal "address" sequence imparts affinity for κ receptors.

Dyn A-(1-13) or Dyn A-(1-11) account for most of the parent peptide's opioid activity.[19] Therefore these two shorter peptides are generally used as the parent peptides for incorporating structural modifications. Modifications such as the substitution of D-Pro in place of Pro at position 10 ([D-Pro^{10}]Dyn A(1-11)-OH, **40**)[104] and incorporation of D-Ala at position 8 ([D-Ala^{8}]Dyn A(1-13)-OH, **41**)[105] have resulted in peptides with enhanced κ receptor affinity and/or selectivity (Table 2.2), and therefore these two compounds have also been used as lead compounds.

Table 2.2: Opioid receptor affinities, selectivity and potencies of selected linear Dyn A analogs

#	Compound	κ Receptor affinity (IC_{50}, nM)	IC_{50}/K_i ratio (κ/μ/δ)	GPI IC_{50} (nM)	Ref
	Dyn A(1-17)-OH	0.23[a]	1/22/11	2.5	107
	Dyn A(1-11)-NH_2	0.077[a]	1/14/91	1.7	107
	Dyn A(1-13)-NH_2	0.023[b]	1/17.4/83	0.24	111
40	[D-Pro[10]]Dyn A(1-11)-OH	0.030[b,d]	1/8/70	0.22	104
41	[D-Ala[8]]Dyn A(1-13)-OH	0.08[a]	1/6/97	1.89	105
42	Ac-Dyn A(1-11)-NH_2	13.2[a]	1/88/189	>3000	107
43	[D-Tyr[1]]Dyn A(1-11)-NH_2	18.6[a]	1/20/65	416	107
44	[Phe[1]]Dyn A(1-11)-NH_2	1.64[a]	1/36/194	1600	107
45	*N*-allyl-[D-Pro[10]]Dyn A(1–11)	0.049[b,d]	1/220/10,180	18.3	110
46	*N*-benzyl-[D-Pro[10]]Dyn A(1-11)	0.029[b,d]	1/1100/6100	990	110
47	*N*-CPM-[D-Pro[10]]Dyn A(1-11)	0.020[b,d]	1/480/28,000	2.16	110
48	*N*,*N*-diallyl[D-Pro[10]]Dyn A(1-11)	3.60[b,d]	1/8.8/41.3	> 3000	110
49	*N*,*N*-dibenzyl[D-Pro[10]]Dyn A(1-11)	3.66[b,d]	1/35/756	228	110
50	*N*,*N*-diCPM[D-Pro[10]]Dyn A(1-11)	0.19[b,d]	1/21/873	>10000	110
51	[D-Asn[2]]Dyn A(1-13)-NH_2	0.0378[b]	1/0.68/29	2.21	111
52	[D-Met[2]]Dyn A(1-13)-NH_2	0.398[b]	1/0.059/54	2.86	111
53	[D-Ser[2]]Dyn A(1-13)-NH_2	0.0568[b]	1/0.38/32	2.22	111
54	[Asn[2]]Dyn A(1-13)-NH_2	16.0[b]	1/6.2/112	469	111
55	[D-Ala[3]]Dyn A(1-11)-NH_2	0.76[a]	1/350/1300	8.1	112
56	[Ala[3]]Dyn A(1-11)-NH_2	1.1[a]	1/190/660	1.7	112
57	[(R)-Atc[4]]Dyn A(1-11)-NH_2	0.89[c]	1/37/>10,000	72.4	113
58	[(S)-Atc[4]]Dyn A(1-11)-NH_2	9.54[c]	1/9.3/>1050	573	113
59	[Nle[6]]DynA(1-11)-NH_2	0.95[a]	1/6.3/1.4	94	107
60	[Nle[7]]DynA(1-11)-NH_2	0.43[a]	1/3.4/7.9	31	107
61	[Lys(Ac)[6]]Dyn A(1-13)-NH_2	0.024[b]	1/0.75/18	0.64	114
62	[Lys(Ac)[7]]Dyn A(1-13)-NH_2	0.14[b]	1/1.3/7.8	0.34	114

[a]Using [^{3}H]U-69,593 as the radioligand. [b]Using [^{3}H]bremazocine as the radioligand. [c]Using [^{3}H]diprenorphine as the radioligand. [d]K_i.

Tyr[1] in Dyn A is the most important residue for κ receptor affinity and activity.[106] Substitution of Tyr[1] with Ac-Tyr (**42**), D-Tyr (**43**), or Phe (**44**) in Dyn A (1-11)-NH_2

resulted in analogs with decreased κ receptor affinity but with higher selectivity towards other opioid receptors (Table 2.2).[107] They also exhibit low potency in functional assays (GPI and MVD). Thus the first residue influences both potency as well as imparting affinity for κ receptors. In another investigation, the N-terminus of [D-Pro^{10}]Dyn A(1-11)-OH was mono- or di-alkylated with allyl, benzyl, and cyclopropylmethyl (CPM) groups.[108-110] All of the *N*-monoalkylated derivatives (**45-47**) exhibit high affinity (K_i < 0.05 nM) for κ receptors in the guinea pig cerebellum, as measured using [^{3}H]bremazocine, and greatly enhanced κ receptor selectivity (> 200-fold over μ and δ receptors) (Table 2.2). These *N*-monoalkylated peptides are among the most selective opioid peptides reported to date. The disubstituted *N*,*N*-diallyl- and *N*,*N*-dibenzylTyr^1-[D-Pro^{10}]Dyn A(1-11) analogs (**48** and **49**) have moderate affinity and selectivity for κ receptors while the *N*,*N*-diCPM analog **50** (Table 2.2) retains high affinity for κ receptors. The *N*,*N*-diCPM analog **50** exhibits negligible agonist activity, and *N*,*N*-diallylTyr^1[D-Pro^{10}]Dyn A(1-11) (**48**) shows weak antagonist activity against Dyn A(1-13)NH_2 in the GPI assay.[110]

The amino acid incorporated in place of Gly^2 of Dyn A affects selectivity and potency.[111] The substitution of Gly with a D-amino acid in Dyn A (1-13)-NH_2 (**51-53**) increases κ opioid receptor affinity; however, these compounds show large increases in μ receptor affinity and therefore exhibit selectivity for μ receptors (Table 2.2). Substitution of Gly^2 with L-amino acids (such as Asn, **54**) is not well tolerated for κ receptors (Table 2.2).[111]

Substitution of the Gly^3 residue of Dyn A has also been investigated.[112] D- and L-Ala substitution of Gly^3 produces the analogs [D-Ala^3]- (**55**) and [Ala^3]Dyn A(1-11)-NH_2

(**56**), which have similar affinities for κ receptors as the parent peptide (Table 2.2) and have enhanced selectivity for κ vs μ and δ receptors (Table 2.2). Analogs with charged residues at this position generally display decreased affinity for κ receptors.[112]

It was hypothesized that the spatial orientation of the aromatic ring at position 4 of Dyn A may be important for opioid receptor affinity and selectivity. A series of [D-Ala^8]Dyn A(1-11)NH_2 analogs with various Phe derivatives (L/D-Homophe (homophenylalanine), (R/S)-Atc (2-aminotetralin-2-carboxylic acid), Aic (2-aminoindane-2-carboxylic acid) and Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid), α-MePhe and N-MePhe) substituted at position 4 were synthesized and evaluated for their opioid receptor affinity and activity.[113] The higher opioid receptor affinity of the (R)-Atc^4 analog (**57**) compared to the (S)-Atc^4 (**58**) (Table 2.2) analog suggests that the χ_1 dihedral angle of Phe^4 in Dyn A most likely adopts a gauche (-) or *trans* conformation in the κ opioid receptor binding site. Changes in the position or orientation of the phenyl ring in this residue do not alter the ability of the peptides to inhibit AC activity.

Residues Arg^6 and Arg^7 are thought to contribute mainly to the selectivity rather than affinity for κ opioid receptors by decreasing μ and δ receptor affinity.[107, 114] Nle (norleucine)- as well as N^{ε}-acetylated lysine-containing analogs (**59-62**) have high affinity for κ receptors, but low selectivity for κ vs μ and δ receptors because they show increased affinity for μ and δ receptors (Table 2.2).

2.3.3 Cyclic Dyn A analogs

Like most other linear peptides, Dyn A can adopt numerous conformations, and the biologically active conformation at κ receptors is not known.[115-118] This inherent

conformational flexibility may be one of the reasons that this peptide also exhibits significant affinity for μ and δ receptors, which leads to low selectivity for κ opioid receptors. Incorporation of a conformational constraint into linear peptides via cyclization can reduce the conformational flexibility;[102] this can be a valuable approach to study the conformational preferences for receptor binding. In addition, cyclization may influence the selectivity of these peptides and improve their metabolic stability and transport across biological membranes.[119] Schwyzer proposed a helical conformation as the bioactive conformation for the N-terminal "message" sequence of Dyn A when bound to opioid receptors.[115] In lipid micelles Dyn A adopts a helical conformation from residues Gly^3 to Arg^9.[118] Based on these observations, several [*i*,*i*+4] and [*i*,*i*+3] cyclic Dyn A analogs have been prepared in attempts to stabilize an α-helical structure.

Cyclizations of Dyn A through disulfide bond formation have been reported (Table 2.3). *cyclo*[D-Cys^2,Cys^5]Dyn A-(1-13) (**63**) was the first conformationally constrained Dyn A analog with high potency in the GPI assay.[120] However, tolerance studies indicated that it interacted mainly with δ receptors and had only minor interaction with κ receptors.[121] In the "address" sequence, a variety of cyclic Dyn A analogs containing disulfide bridges were also made (for example compounds **64-68**, see Table 2.3).[122-124] In guinea pig brain, these analogs display high binding affinity for both κ and μ receptors, resulting in low selectivity for κ opioid receptors.

Table 2.3: Opioid receptor affinities, selectivity and potencies of selected cyclic Dyn A analogs

#	Compound	κ receptor affinity (IC_{50}, nM)	IC_{50}/K_i ratio (κ/μ/δ)	Potency IC_{50} (nM, GPI)	Ref
	Dyn A(1-11)-NH_2	0.077[a]	1/22/11	7.5	122
64	*cyclo*[Cys^5,Cys^{11}] Dyn A(1-13)-NH_2	0.285[a]	1/0.95/5.7	1080	122
65	*cyclo*[Cys^8,Cys^{13}] Dyn A(1-13)-NH_2	0.074[a]	1/13/54	1.3	122
66	*cyclo*[Cys^5,Cys^9] Dyn A(1-11)-NH_2	0.86[a]	1/7.4/36	411	123
67	*cyclo*[Cys^5,Cys^{10}] Dyn A(1-11)-NH_2	0.59[a]	1/7.2/139	286	123
68	*cyclo*[Cys^6,Cys^{10}] Dyn A(1-11)-NH_2	0.05[a]	1/1.9/10	6.51	123
	Dyn A(1-11)-NH_2	0.58[a]	1/17.1/45	1.07	125
69	*cyclo*[D-Asp^2,Lys^6] Dyn A(1-11)-NH_2	110[a]	1/0.03/0.11	610	125
70	*cyclo*[D-Asp^3,Lys^7] Dyn A(1-11)-NH_2	4.9[a]	1/64/68	600	125
71	*cyclo*[Lys^5,D-Asp^9] Dyn A(1-11)-NH_2	6.6[a]	1/1.8/7.6	2500	125
	Dyn A(1-13)-NH_2	0.15[b,d]	1/1.3/26	0.22	126
72	*cyclo*[D-Asp^2,Dap^5] Dyn A(1-13)-NH_2	0.22[b,d]	1/2.2/46	0.16	126
73	*cyclo*[D-Asp^2,Dab^5] Dyn A(1-13)-NH_2	0.13[b,d]	1/1.3/92	0.23	126
74	*cyclo*[D-Asp^2,Orn^5] Dyn A(1-13)-NH_2	0.68[b,d]	1/0.28/21	0.40	126
	Dyn A(1-13)-NH_2	0.31[c,d]	1/3/92	0.19	127
75	*cyclo*[D-Asp^5,Dap^8] Dyn A(1-13)-NH_2	8.0[c,d]	1/9.4/410	>5000	127
76	*cyclo*[D-Asp^6,Dap^9] Dyn A(1-13)-NH_2	2.6[c,d]	1/1.7/19	46	127

[a]Using [^{3}H]U-69,593 as the radioligand. [b]Using [^{3}H]bremazocine as the radioligand. [c]Using [^{3}H]diprenorphine as the radioligand. [d]K_i.

Cyclizations of Dyn A through lactam bond formation have also been reported. In one series of *cyclo*[*i*,*i*+4] compounds, positions 2 and 6, 3 and 7, and 5 and 9 were used as the sites for incorporating cyclic conformational constraints (**69-71**, Table 2.3).[125] Cyclization between D-Asp^2 and Lys^6 in *cyclo*[D-Asp^2,Lys^6]Dyn A(1-11)-NH_2 (**69**)

resulted in high potency and selectivity for μ opioid receptors, whereas the cyclization between D-Asp^3 and Lys^7 led to a potent ligand *cyclo*[D-Asp^3,Lys^7]Dyn A(1-11)-NH_2 (**70**) with the highest κ opioid receptor affinity and selectivity in the series. *cyclo*[Lys^5,D-Asp^9]Dyn A(1-11)-NH_2 (**71**) shows high affinity at κ receptors, but this compound also exhibits similar affinity for μ receptors, and therefore this compound is nonselective.

In one series of [2,5] cyclic compounds, Dap (2,4-diaminopropionic acid), Dab (2,4-diaminobutyric acid), and Orn at position 5 were cyclized with D-Asp^2 to create 4-, 5-, and 6-atom bridges between the two α-carbons (peptides **72-74**), respectively (Table 2.3).[126] All of the cyclic analogs have high affinity for κ and μ receptors and high potency in the GPI assay. However, as the ring size increased, the selectivity for κ receptors decreased and the peptides became selective for μ receptors (Table 2.3). Compounds **72-74** had comparable potency in the GPI to Dyn A(1-13)NH_2.

Cyclization in the "address" sequence of Dyn A has also been explored utilizing positions 5 and 8, and 6 and 9 to make lactam bridges (compounds **75** and **76**, Table 2.3).[127] The *cyclo*[5,8] analog **75** has lower κ receptor affinity, but moderate κ vs μ receptor selectivity. The *cyclo*[6,9] analog **76** shows high affinity for κ receptors, but the analogs are nonselective for κ receptors.

2.3.4 Kappa receptor antagonists derived from Dyn A

In recent years, a number of opioid receptor antagonists derived from Dyn A have been discovered (Table 2.4). Tyr^1 is important for both the affinity and efficacy of Dyn A. Substitution Phe in position 1 of [D-Ala^8]Dyn A(1-11)NH_2 resulted in a Dyn A analog **77** reported to exhibit very low potency in the GPI (Table 2.4).[107] Compounds with a

bulky group on the α-amino group such as *N*,*N*-diallyl (**48**) and *N*,*N*-diCPM (**50**) of Tyr[1] are antagonists that also show high affinity and selectivity for the κ receptors (Table 2.2).[108-110] Dynantin (**38**, Figure 2.5), with the Tyr residue replaced by the des-amino tyrosine derivative (2S)-Mdp ((2*S*)-2-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid), shows high κ receptor affinity, selectivity, and antagonist potency.[89]

Substitution with the constrained amino acid Pro at this position of Dyn A-(1-11)NH_2 resulted in the peptide [Pro^3]Dyn A-(1-11)NH_2 (**78**) with the highest κ receptor selectivity among the analogs with modification in this position.[128] However, the antagonist activity of this peptide at κ opioid receptors is very weak.[128]

Chimeric Dyn A analogs have been designed in our laboratory as κ opioid receptor antagonists. Attachment of an acetyl-protected tetrapeptide (Ac-Tyr-Lys-Trp-Trp-NH_2) that has weak antagonist activity at κ receptors[129] to the C-terminal "address" sequence of [D-Ala^8]Dyn A(1-11)-NH_2 resulted in the novel acetylated Dyn A analog JVA-901 (Ac[Lys^2,Trp^3,Trp^4,D-Ala^8]Dyn A-(1-11)NH_2, **79**).[130] This compound exhibits antagonist activity and enhanced affinity for κ opioid receptors compared to the tetrapeptide.[130]. Replacement of the N-terminal "message" sequence of [D-Ala^8]Dyn A(1-11)NH_2 with a small μ opioid receptor antagonist followed by structural modifications using a combinatorial library resulted in the discovery of arodyn (**39**), a potent and highly selective antagonist at κ receptors.[90]

Cyclic Dyn A analogs with antagonist activities have also been reported. Based on the novel acetylated chimeric Dyn A analog JVA-901 (**79**), our laboratory designed and synthesized cyclodyn (*cyclo*^{N,5}[Trp^3,Trp^4,Glu^5]Dyn A-(1-11)NH_2, **80**), the first cyclic

Dyn A analog with antagonist activity at κ opioid receptors.[131] Very recently, our research group identified a Dyn A analog cyclized in the C-terminal "address" domain, [N^{α}-benzyl-Tyr^{1},*cyclo*(D-Asp^{5},Dap^{8})]Dyn A-(1-11)NH_2 (**81**), as a novel κ opioid receptor antagonist.[132]

Table 2.4: Opioid receptor affinities, selectivity and potencies of selected Dyn A analogs with antagonist activity

#	Compound	K_i (κ, nM)	K_i ratio (κ/μ/δ)	Antagonist potency	Ref
38	Dynantin	0.823[a]	1/259/198	0.632[c]	89
39	Arodyn	10.0[b]	1/174/583	2.0[d]	90
78	[Pro^{3}]Dyn A(1-11)-NH_2	2.7[b]	1/2110/3260	380[e]	128
79	JVA-901	19.8[b]	1/12.6/268	f	130
80	Cyclodyn	27[b]	1/12/330	f	131
81	[N^{α}-benzyl-Tyr^{1}, *cyclo*(D-Asp^{5},Dap^{8})] Dyn A-(1-11)NH_2	30[b]	1/194/330	84[d]	132

[a]Using [^{3}H]U-69,593 as the radioligand. [b]Using [^{3}H]diprenorphine as the radioligand. [c]K_e. [d]K_B. [e]IC_{50}. [f]Reverses the agonist activity of Dyn A(1-13)NH_2.

2.4 Conclusions

Since their discovery, extensive research has been focused on the study of the physiological and pharmacological roles of opioid receptors and their ligands. The research has resulted in the identification of numerous potent analgesics that mainly act on μ opioid receptors. However, these analgesics generally have severe side effects. Peripherally acting κ opioid receptor agonists can act as analgesics with anti-inflammatory effects[5] while lacking the CNS side effects of both μ opioid receptor agonists and centrally-acting κ opioid receptor agonists (e.g. dysphoria).

Kappa agonists have potential therapeutic applications in several other areas in addition to pain relieve. Kappa opioid receptor agonists have potential for the treatment

of cocaine addiction[63] and for the treatment of HIV-1 and HIV-1 related encephalopathy.[65, 66] On the other hand, κ opioid receptor antagonists have the potential to be used therapeutically in the treatment of cocaine[68, 69] and opioid dependence[70, 71] and have been shown to have antidepressant activity.[67] Therefore there is great interest in studying κ opioid receptor ligands as pharmacological tools and potential therapeutic agents.

Dyn A is the best studied endogenous ligand[92] for κ opioid receptors and is involved in a variety of physiological and pharmacological functions.[94, 95, 99-101] The SAR of this peptide has been explored and thus has resulted in a variety of analogs, some with improved affinity, selectivity, and/or potency.[5, 102, 103]

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Chapter 3. Deletion of Ac-NMePhe^{1} from [NMePhe^{1}]arodyn under Acidic Conditions: Effects of Cleavage Conditions and N-Terminal Functionality

*Note that the compound numbers used in this chapter are only applicable within this specific chapter.

3.1 Introduction

There has been a significant increase recently in the study of N-methylated peptides.[1-3] N-Methylated peptides have been isolated from a variety of natural sources including plants, marine animals and microoraganisms.[4, 5] N-Methylation can greatly increase metabolic stability towards peptidases,[6, 7] and elimination of the hydrogen-bond donor can increase lipophilicity and thereby potentially increase bioavailability.[8] Cyclosporines, natural products produced by the fungus *Beauveria nivea* which contain multiple N-methylamino acids, have anti-fungal, anti-inflammatory and immunosuppressive activities.[2, 9, 10] Cyclosporine A, an undecapeptide with seven N-methylamino acids, was found to be orally active, probably due to N-methylation and intramolecular hydrogen bonds, even though it has a relatively high molecule weight. Therefore, N-methylated peptides are increasingly of interest as potentially useful therapeutics. N-Methylamino acids are also of interest because of their conformational effects on peptides.[1, 6, 7] The proportion of the *cis* conformation of the amide bond is significantly higher when the amide nitrogen is methylated,[1, 11, 12] and elimination of a hydrogen-bond donor can also affect the peptide secondary structure. More recently, N-methylated cyclic pentapeptides have been used as templates for the rational design of peptides with distinct backbone conformations.[13]

Our research focuses on the development of potent and highly selective peptide antagonists for kappa (κ) opioid receptors and examination of the structure-activity relationships (SAR) for antagonist activity at these receptors. In addition to their use as pharmacological tools, κ opioid receptor antagonists have the potential to be used therapeutically in the treatment of cocaine[14, 15] and opioid dependence[16, 17] and have been

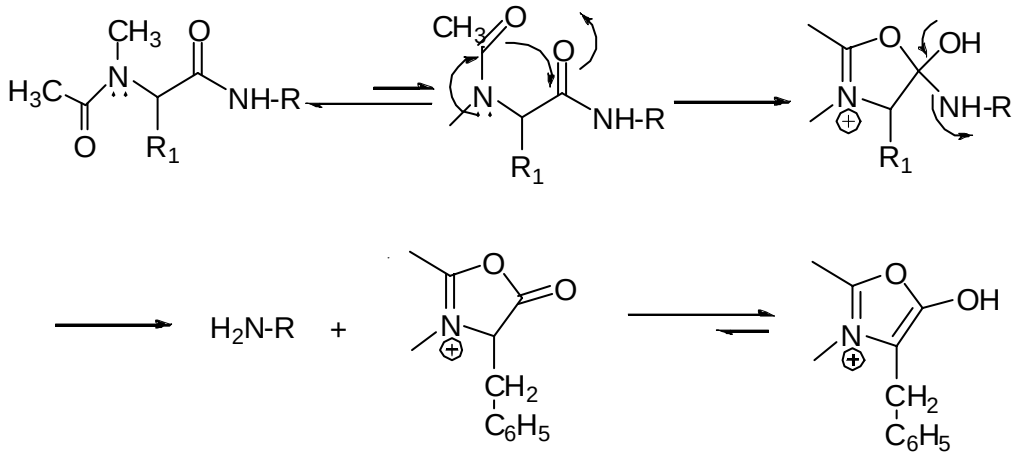
shown to have antidepressant activity.[18] Dynorphin A (Dyn A, Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln) is proposed to be an endogenous ligand for κ opioid receptors.[19] Arodyn (Ac[Phe^{1,2,3},Arg^{4},D-Ala^{8}]Dyn A(1-11)NH_2, **1**) is an acetylated Dyn A analog identified in our laboratory that is a potent and highly selective κ opioid receptor antagonist,[20] and its analog [NMePhe^{1}]arodyn (**2**) shows even higher affinity and selectivity for κ opioid receptors.[21]

During the synthesis of **2**, two major products were obtained and characterized by high-performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS). Mass spectral analysis of the mixture of products showed the desired peptide ($[M+2H^+]^{2+}$ = 774.9) and another dominant product ($[M+2H^+]^{2+}$ = 673.8) corresponding to arodyn(2-11) with the loss of the Ac-NMePhe moiety (mass = 203.7) from the desired peptide.[22] Cleavage of an aliquot of the peptide from the resin prior to acetylation indicated that the deletion was not due to incomplete coupling. Because of the remarkable pharmacological activity of [NMePhe^{1}]arodyn, this side reaction was studied in detail.

Even though there were several reports of a similar side reaction involving trialkyl glycine derivatives in both linear and cyclic peptides,[23-26] there was only one recent report on a similar side reaction involving an Ac-N-methyl-α-monoalkyl-amino acid.[27] The mechanistic aspects of this side reaction have been studied (Scheme 3.1).[23] The carbonyl oxygen of the acetyl group acts as a nucleophile and attacks the adjacent carbonyl group to form a five-membered oxazolinium intermediate, releasing the rest of the peptide. For N-methylamino acids with an α-hydrogen, the oxazolinium intermediate can undergo tautomerization to form a stabilized aromatic ring. Here we describe our efforts to

optimize the cleavage conditions to improve the yields of the Ac-N-methyl peptides and the synthesis of stable analogs that do not undergo this side reaction.

Scheme 3.1: Proposed mechanism for the deletion of Ac-N-methyl-α-monoalkyl-amino acid from peptides under acidic conditions

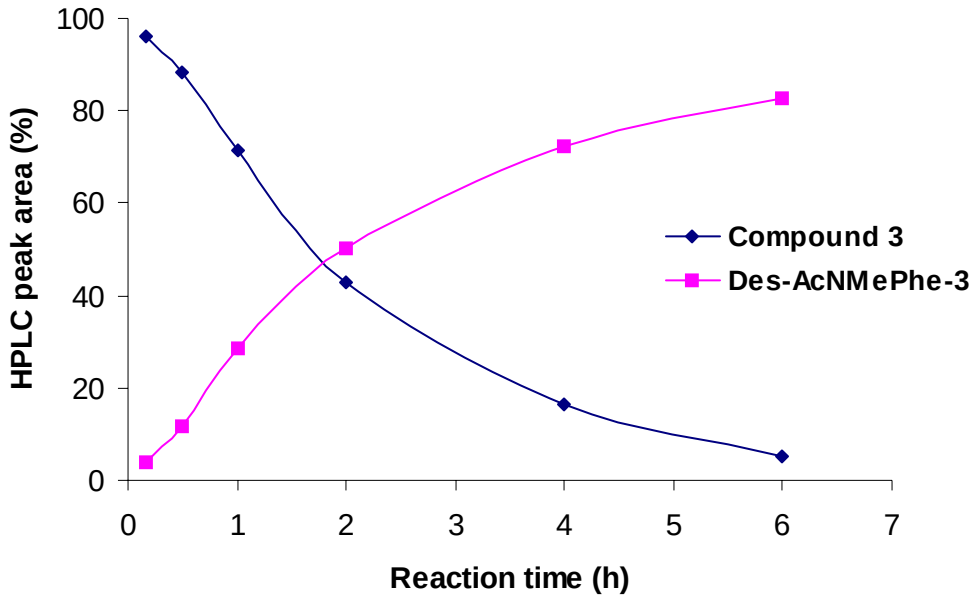


(R = the rest of the peptide)

3.2 Results and discussion

[NMePhe[1],Trp[3]]arodyn, **3** (Table 3.2), was chosen as the parent compound for these studies since this peptide has strong UV absorbance at 280 nm, facilitating monitoring this side reaction. Pure **3** underwent degradation under standard cleavage conditions (2 h, Reagent B, TFA:H_2O:phenol:triisopropylsilane (TIPS) = 88:5:5:2,[28] Figure 3.1). Within 2 h, the typical time used for cleavage of peptides from the resin, approximately 60% of the peptide was degraded. Almost all of the parent peptide had decomposed after 6 h at room temperature. [Trp[3]]arodyn-(2-11) was the major product obtained.

Figure 3.1: Degradation of pure **3** in Reagent B at room temperature



The yield for the desired peptide **3** following cleavage from the resin was only about 8% under standard cleavage conditions (Reagent B, 2 h at room temperature, Figure 3.2). Different cleavage cocktails at lower temperature (4 ^{o}C) were evaluated to minimize the side reaction and to maximize the yield of pure **3** (Table 3.1). Incomplete deprotection of the amino acid side chain protecting groups (i.e. the Pbf group in Arg) was observed under all of the examined conditions, presumably due to the lower cleavage temperature and resulting slower deprotection reactions. Interestingly, cleavage of the peptide by pure TFA for 3 h gave the highest yield (44%) of **3** among the different cleavage conditions. The addition of water as a scavenger significantly decreased the relative amount of the desired peptide obtained as determined by HPLC.

Table 3.1: Effects of different cleavage conditions and reaction times at 4 ^{O}C on the yield of **3**, as measured by HPLC

Cleavage Cocktail	Cleavage Time (h)	% Area of **3**
Reagent B[a]	2 3.5	17 25
Pure TFA	1 2 3 4	37 35 44 35
TFA: water = 95:5	1 2 3	8 11 22

[a]TFA:phenol:water:TIPS = 88:5:5:2

Since the acetyl group as well as the first amino acid (NMePhe) was proposed to be involved in the deletion reaction, we designed arodyn analogs in which the acetyl group of **3** was either deleted (compound **4**) or replaced with an alternative group, or the NMePhe was substituted with Phe (compound **5**) or Tic (Tic = 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, peptide **6**, Table 3.2). Compounds **7-10** (Table 3.2) are acylated with heteroatom containing groups, while peptides **11-17** (Table 3.2) are acylated with a variety of acids, some of which are quite sterically hindered. All of these analogs were synthesized using standard solid-phase peptide synthesis (SPPS) and cleaved using Reagent B at room temperature for 2 h. HPLC and ESI-MS analysis were used to identify the desired peptides and the deletion product. Retention times (t_R) of the corresponding desired peptides are shown in Table 3.2. The percent peak area in the HPLC chromatograms for the desired peptide and the (2-11) deletion product are shown in Figure 3.2.

Table 3.2: Structures and retention times (t_R) of **3** and its analogs

X-NMePhe-Phe-Trp-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2

#	X	t_R (min)[a]	#	X	t_R (min)[a]	#	X	t_R (min)[a]
3	Ac	24.9	**8**	$PhOCH_2CO$	30.2	**13**	Piv	24.7
4	H	18.1	**9**	Gly	19.0	**14**	$C_6H_5CH_2CO$	30.1
5[b]	Ac-Phe[1]	22.7	**10**	Ac-Gly	22.6	**15**	C_6H_5CO	29.3
6[b]	Ac-Tic[1]	24.0	**11**	CH_3CH_2CO	NA[c]	**16**	2-MeO-C_6H_4CO	27.7
7	CH_3OCO	25.5	**12**	$(CH_3)_2CHCO$	26.3	**17**	2,6-DiMeO-C_6H_3CO	28.6

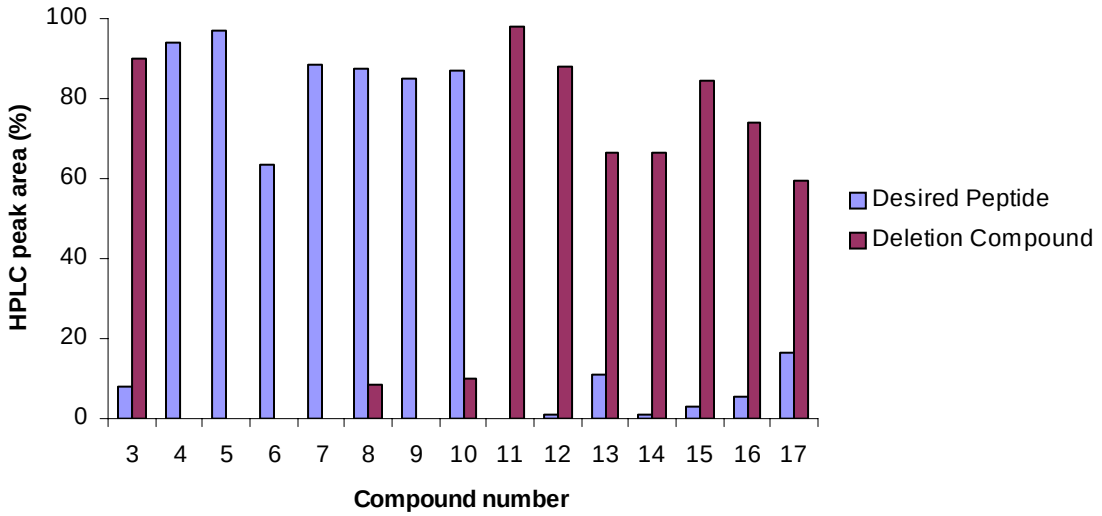
[a]HPLC conditions: 5-50% acetonitrile containing 0.1% TFA over 45 min at 1 mL/min; the retention time of [Trp[3]]arodyn (2-11) is 15.4 min. [b]First amino acid is Phe or Tic instead of NMePhe. [c]No product was observed under standard cleavage conditions.

As expected based on the mechanism, the des-acetyl analog **4** (Table 3.2) gave the expected peptide without any evidence of the deletion sequence (Figure 3.2), verifying the important role of the acetyl group in the deletion reaction. When the first amino acid residue is Phe (compound **5**) instead of NMePhe, no deletion product was detected. Thus the N-methyl group is required for the deletion reaction to occur, most likely due to changing the backbone conformation to one that favors the cyclization. When the first amino acid is Tic (compound **6**), a conformationally constrained NMePhe analog, no deletion side product was detected. This suggests that the formation of the 5-membered ring intermediate does not occur for this constrained N-alkyl amino acid.

Little to no deletion sequence was detected following cleavage of peptides **7**-**10** from the resin (Figure 3.2). Groups attached to the N-terminus that are heteroatom containing can decrease the electron density on the carbonyl and thus make the oxygen less nucleophilic, preventing the side reaction. An oxygen atom attached to or separated by one methylene from the carbonyl (peptides **7** and **8**, respectively) both have a similar effect, although a small portion of deletion product (8%) was observed for peptide **8**. The

methoxyl group in **7** can act both as an electron-withdrawing group by field effect and an electron-donating group by resonance effect. In this situation, the field effect appears to outweigh the resonance effect. This is similar to one report where the carbamate group is not as a good hydrogen bond acceptor as a tertiary amide,[29] presumably also due to the electron-withdrawing effect outweighing the resonance effect. When glycine was coupled to the N-terminus (peptide **9**), no deletion sequence was observed, most likely due to the electron-withdrawing effect of the protonated amine. When the amine was acetylated (peptide **10**), however, a small amount of the deletion sequence (10%) was observed, similar to the results for peptide **8**. This is most likely due to the decreased electron-withdrawing effect of the acetylated amine. This also demonstrates that the peptide is reasonably stable when the N-methylamino acid is not at the N-terminus of a peptide.

Figure 3.2: Desired and deletion products for peptide **3** and its analogs following cleavage from the resin[a]



[a]Cleavage conditions: Reagent B for 2 h at room temperature.

The acyl groups incorporated in peptides **11-17**, however, could not prevent the side reaction, and thus the deletion sequence was the major product found following cleavage of these peptides from the resin (Figure 3.2). The two most sterically hindered analogs, where the acetyl group was substituted with Piv (pivaloyl, 2,2,2-trimethylacetyl) and 2,6-dimethoxylbenzoyl groups (compounds **13** and **17**, respectively), yielded similar amounts of the desired peptide (11% and 17%, respectively) as **3** (8%).

3.3 Conclusions

In conclusion, we described the efforts toward minimizing the side reaction involving deletion of an acetylated N-methylamino acid from the N-terminus of a peptide. In order to obtain higher yields of these peptides, alternative cleavage conditions were explored. While alternative cleavage conditions increased the yields of the full-length peptide, the deletion reaction still occurred for the acetylated peptides, decreasing the yield of the desired peptide. Substitution of the N-terminal acetyl group with different electron-withdrawing groups (for example, a methoxycarbonyl group and amino acid) was found to prevent the side reaction. Other acyl groups, including those with bulky substituents, on the other hand, did not prevent the deletion reaction. The pharmacological effects of these substitutents on κ opioid receptor affinity, selectivity and efficacy are described in the next chapter.

3.4 Experimental section

3.4.1 Materials

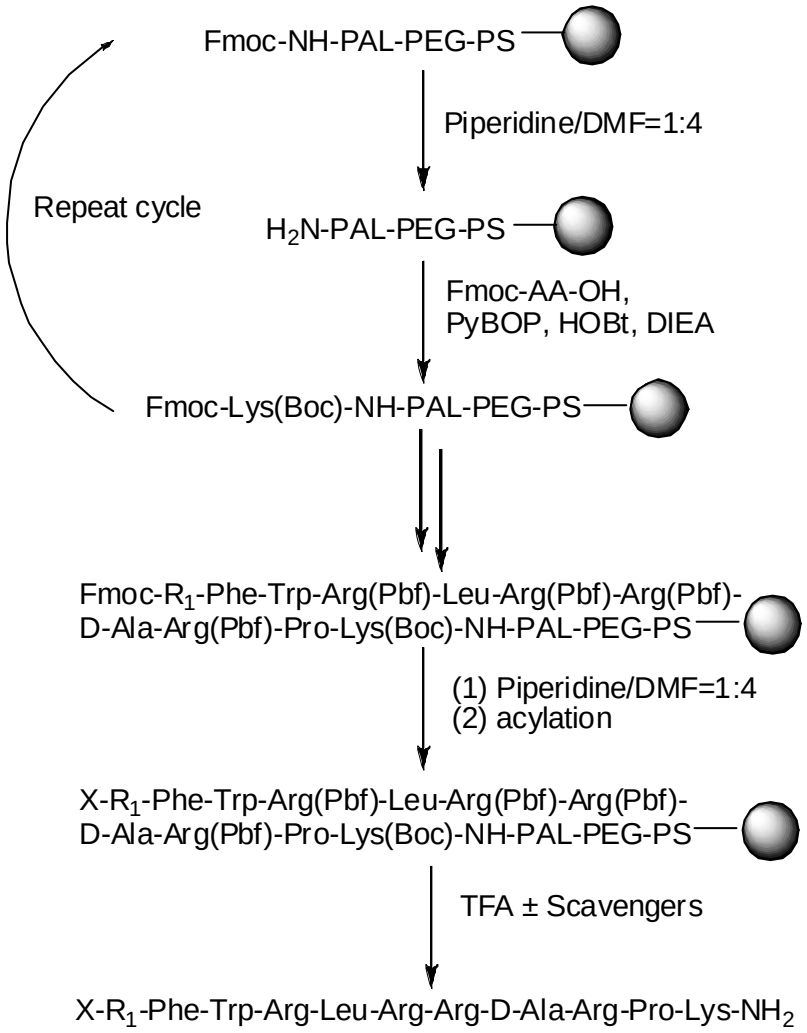
All Fmoc-protected (Fmoc = 9-fluorenylmethoxycarbonyl) amino acids were purchased from Bachem (King of Prussia, PA), Calbiochem-Novabiochem (San Diego, CA), Applied Biosystems (Foster City, CA), or Peptides International (Louisville, KY). Fmoc-PAL-PEG-PS resin (PAL-PEG-PS = peptide amide linker-poly(ethylene glycol)-polystyrene) was purchased from Applied Biosystems. Benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) was purchased from Calbiochem-Novabiochem. Dichloromethane (DCM), *N*,*N*-diisopropylethylamine (DIEA), *N*,*N*-dimethylformamide (DMF), acetic acid, diethyl ether, acetonitrile, methanol, and trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Hampton, NH). 1-Hydroxylbenzotriazole (HOBt) and triisopropylsilane (TIPS) were purchased from Acros Chemical Co. (Pittsburgh, PA). All other chemicals including phenol, piperidine, acetic anhydride, methyl chloroformate, propionic anhydride, isobutyric anhydride, trimethylacetic anhydride, phenoxyacetic chloride, phenylacetyl chloride, benzoyl chloride, *o*-anisoyl chloride, and 2,6-dimethoxylbenzoyl chloride were purchased from Aldrich Chemical Co. (Milwaukee, WI).

3.4.2 Peptide synthesis

All peptides were synthesized on the PAL-PEG-PS resin (0.16 or 0.24 mmol/g, 200 mg) by SPPS using Fmoc-protected amino acids according to standard procedures (Scheme 3.2). The resin was first swollen with 10 mL of DCM/DMF (1:1, 2 X 10 min). The desired Fmoc-protected amino acids were coupled to the growing peptide chain with

PyBOP, HOBt, and DIEA (4/4/10 relative to the resin substitution) in DMF (2 mL) for 2 h. Completion of the coupling reactions was determined by the ninhydrin and/or the chloranil tests.[30, 31] The side chains of Lys and Trp were protected by Boc (*tert*-butyloxycarbonyl), and Arg by Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl). Following the coupling reaction, the resin was washed with 10 mL of DCM/DMF (1:1, 5 X 30 sec). The Fmoc group was then removed using 20% piperidine in DMF (5 mL, 2 X 10 min), the resin washed with DCM/DMF (1:1, 10 X 30 sec) and the next amino acid then coupled to the resin. After completion of the peptide assembly and removal of the Fmoc group from the N-terminal residue, different acyl groups were attached to the N-terminal free amine except for the peptides **4** and **9**, which have a free amine at the N-terminus. For the synthesis of **3**, **5**, **6** and **10**, acetic anhydride (20 equiv) in DMF (2 mL) was reacted with the resin for 30 min. The approximate acid anhydrides (20 equiv in DMF) were used to synthesize **11**-**13**. The corresponding acid chlorides (20 equiv) with an equiv amount of DIEA in DMF (2 mL) were used to prepare **7**, **8**, and **14**-**17**. The resins were then washed successively with DCM/DMF (1:1, 5 X 30 sec), and finally with methanol. The resins were dried under vacuum before cleavage.

Scheme 3.2: SPPS of arodyn analogs



3.4.3 Cleavage of the peptides from the resin under standard conditions

The peptides were cleaved from the resin with 5 mL of Reagent B (88% TFA, 5% phenol, 5% water, and 2% TIPS) at room temperature for 2 h.[28] The solutions were then filtered from the resin and washed with TFA (1 mL). The solutions were diluted with 10% acetic acid (30 mL) and extracted with ether (2 X 30 mL), and the ether extracts then back extracted with acetic acid (2 X 10 mL). Residual ether in the combined

aqueous solutions was evaporated under vacuum, and the solutions were lyophilized to give the crude peptides.

3.4.4 Cleavage of peptide 3 under different cleavage conditions at 4 ^{o}C

Protected peptide **3**-resin (80-110 mg) was mixed with 1 mL of one of three different cleavage reagents (pure TFA, Reagent B, or 95% TFA with 5% water) for 1 to 4 h at 4 ^{o}C, as shown in Table 2.1. Aliquots (0.2 mL) of the cleavage reactions were removed at various times and diluted with 2 mL water. After filtering, the filtrates were frozen, lyophilized, dissolved in water and analyzed by HPLC using a gradient of aqueous MeCN containing 0.1% TFA as described below.

3.4.5 Treatment of purified peptide 3 with Reagent B at room temperature

Purified peptide **3** (2.23 mg) was mixed with 1 mL of reagent B, and aliquots of the reaction mixture (0.1 mL) were removed at various time points and diluted with H_2O (1 mL). The aliquots were frozen, lyophilized, dissolved in water and analyzed by HPLC.

3.4.6 HPLC and ESI-MS analysis

All of the crude peptides were analyzed by HPLC. A linear gradient of 5-50% solvent B (solvent A aqueous 0.1% TFA, and solvent B MeCN containing 0.1% TFA) over 45 min, at a flow rate of 1 mL/min, was used for analysis. The UV wavelength of 214 nm was used to quantify the yields of the desired peptides and deletion product. Peptide **3** has a retention time of 24.9 min, while the deletion product [Trp^{3}]arodyn (2-11)

has a retention time of about 15.4 min. The molecular weights of the peptides were determined by ESI-MS (Waters, Q-TOF analyzer).

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Chapter 4. Deletion of Ac-NMePhe^{1} from [NMePhe^{1}]arodyn under Acidic Conditions: Effects of Substitutions on Pharmacological Activity

*Note that the compound numbers used in this chapter are only applicable within this specific chapter.

4.1 Introduction

Agonists at opioid receptors, such as morphine and its derivatives which activate mu (μ) opioid receptors, have been used extensively as narcotic analgesics.[1] However, their use is associated with serious side effects, namely respiration depression, psychological and physical dependence, and constipation. Therefore there is great interest in developing agents for the other opioid receptors, namely kappa (κ) and delta (δ) opioid receptors. Compared with μ agonists, κ opioid receptor agonists lack these side effects, although they generally show different side effects such as dysphoria.[2, 3] Kappa opioid agonists can produce analgesic activity peripherally both in somatic and visceral pain models, particularly in certain conditions involving inflammation.[4] Peripherally acting κ receptor agonists do not have the CNS side effects, and therefore there has been an interest in developing peripheral κ agonists.[1]

Kappa receptor antagonists were initially mainly used as pharmacological tools to study receptor function and physiological and pharmacological actions of κ receptor agonists. Recently, κ antagonists were demonstrated to have antidepressant activity.[5] They also have potential use in the treatment of cocaine [6, 7] and opioid dependence.[8, 9]

A number of κ opioid receptor antagonists have been discovered during the past two decades. The bivalent ligand nor-binaltorphimine (nor-BNI) was identified as a potent and selective κ opioid antagonist.[10] Extensive structure-activity relationship (SAR) studies of the δ opioid receptor antagonist naltrindole resulted in the identification of 5'-guanidinyl naltrindole (5'-GNTI) as a κ opioid receptor selective antagonist.[11] JDTic is the first phenylpiperidine derived compound that is a potent and selective antagonist for κ opioid receptors.[12] It shows very high affinity (K_i = 0.006 nM) and selectivity (570- and >16,000-fold selectivity over μ and δ opioid receptors, respectively) for κ opioid receptors. However,

all three antagonists exhibit extremely long half-lifes (ranging from several days to several weeks),[13-16] thus limiting their usage as pharmacological tools.

The endogenous ligands for κ opioid receptors are peptides. Therefore, we are exploring the SAR of the endogenous peptide Dyn A (Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln), with an emphasis on analogs that are selective κ opioid receptor antagonists. It has been proposed that peptide and nonpeptide ligands bind to different domains of the κ opioid receptor.[17-19] Therefore, selective peptide antagonists are complementary to nonpeptide antagonists. Furthermore, peptide derivatives are expected to have shorter half-lifes due to metabolism by peptidases, and therefore peptide antagonists could overcome problems associated with long-acting nonpeptide antagonists.

Derivatives of Dyn A with antagonist activity have been reported.[1] Early analogs generally exhibited weak antagonist activity and low κ opioid receptor selectivity, but recently more selective and potent analogs have been reported. [Pro^3]Dyn A-(1-11)NH_2 has high affinity and selectivity for κ opioid receptors, but this ligand exhibits only weak antagonist potency in functional assays.[20] Dynantin ([(2S)-Mdp^1]Dyn A-(1-11)NH_2, Mdp = 2-methyl-3-(2',6'-dimethyl-4-hydroxyphenyl)propionic acid), with modification of the first amino acid Tyr, was reported to be a potent κ antagonist with high binding affinity and selectivity for κ opioid receptors.[21]

Our laboratory has identified several κ opioid receptor antagonists by modifying the N-terminal "message" sequence[22] of Dyn A. These include three peptides lacking a basic N-terminal amine.[23-25] One of these peptides is arodyn (**1**), a potent (K_i (κ) = 10 nM) and highly selective (K_i ratio (κ/μ/δ) = 1/174/583)[24] antagonist for κ opioid receptors (K_B = 2 nM, Bennett, Murray and Aldrich, unpublished results). SAR studies of arodyn resulted in [NMePhe^1]arodyn (**2**) that exhibits higher affinity (K_i (κ) = 4.56 nM) than arodyn and very high selectivity (K_i ratio (κ/μ/δ) = 1/1100/>2170) for κ opioid receptors.[26] This is by far one

of the most selective peptide antagonists for κ opioid receptors. Another peptide antagonist reported with extremely high κ opioid receptor selectivity is [Pro^3]Dyn A(1-11)NH_2 (K_i ratio (κ/μ/δ) = 1/2110/3260).[20]

However, it was found that [NMePhe^1]arodyn and its analogs that are acetylated at the N-terminus are all prone to loss of Ac-NMePhe under acidic cleavage conditions (see Chapter 3). The yields of these peptides could be increased by modifying the cleavage cocktail and cleavage conditions, but even under optimized cleavage conditions, substantial degradation of these peptides still occurred. We showed that this degradation reaction could be suppressed by substituting the acetyl group in [NMePhe^1,Trp^3]arodyn (**3**) with a heteroatom-containing group (e.g. methoxycarbonyl, phenyloxyacetyl, glycyl, and acetyl glycyl; see Chapter 3). Considering the remarkable pharmacological activity of [NMePhe^1]arodyn, the pharmacological profiles of these stable analogs were examined. A previous SAR of arodyn revealed that Phe^3 is an important residue in the N-terminal "message" sequence for κ opioid receptor affinity, selectivity, and efficacy.[26] Since the substitution of Phe^1 with NMePhe can significantly increase κ opioid receptor affinity and selectivity, other stable [NMePhe^1]arodyn analogs in which position 3 was substituted with other aromatic or non-aromatic residues were also evaluated for κ opioid receptor affinity, selectivity, and opioid activity.

4.2 Results and discussion

4.2.1 Analog design

We designed the following analogs for evaluation of their pharmacological activities. First, we evaluated the effects of substituting different heteroatom-containing groups using [NMePhe^1]arodyn (**2**) and [NMePhe^1,Trp^3]arodyn (**3**) as the parent compounds. The Phe^1 analog of **3** (**4**) was also prepared to evaluate the effects of N-methylation on κ receptor

affinity and selectivity. Thus, we synthesized [CH_3OCO-NMePhe^1,X^3]arodyn (**5** and **9**), [$PhOCH_2CO$-NMePhe^1,X^3]arodyn (**6** and **10**), [Gly-NMePhe^1,X^3]arodyn (**7** and **11**), and [Ac-Gly-NMePhe^1,X^3]arodyn (**8** and **12**) (X = Phe or Trp, Table 4.1). Compounds **3**, **4**, and **9-12** were originally synthesized for the study of the side reaction (see Chapter 3). The methoxycarbonyl group was also incorporated in analogs where the third position was substituted with other aromatic residues (Tyr and 2-naphthylalanine (Nal(2')) in compounds **13** and **14**, respectively) or a non-aromatic residue (cyclohexylalanine (Cha) in compound **15**). Their corresponding unstable acetyl analogs (compounds **16-18**, Table 4.1) had been synthesized previously for comparison of their κ opioid receptor affinity, selectivity and efficacy (Bennett, Murray and Aldrich, unpublished results).

4.2.2 Chemistry

All peptides were synthesized on the Fmoc-PAL-PEG-PS (PAL-PEG-PS = peptide amide linker-poly(ethylene glycol)-polystyrene) resin by solid-phase peptide synthesis (SPPS) using Fmoc-protected amino acids according to standard procedures.[24, 26] After completion of the peptide assembly and removal of the Fmoc group from the N-terminal residue, different acyl groups were coupled to the N-terminal free amine. After synthesizing the protected peptides on the resin, these peptides were cleaved with Reagent B (88% trifluoroacetic acid (TFA), 5% phenol, 5% water, and 2% triisopropylsilane (TIPS))[27] or pure TFA (for compounds **2**, **3**, and **16-18**, see Chapter 3) and purified by reversed-phase high-performance liquid chromatography (HPLC). The purity of the final purified peptides was generally >98% as verified by two different HPLC solvent systems (MeCN/water and MeOH/water containing 0.1% TFA). The molecular weights of these peptides were verified by electrospray ionization mass spectrometry (ESI-MS). The analytical data for the peptides is presented in Table 4.1.

Table 4.1: Analytical data for the peptides

X-NMePhe-Phe-R_1-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2

Cmpd	X	R_1	HPLC (t_R, (min)/purity)		ESI-MS (*m/z*)	
			System 1[a]	System 2[b]	Calculated	Observed
3	Ac	Trp	24.65/100	41.06/100	$[M+3H]^{3+}$=530.0 $[M+2H]^{2+}$=795.0	$[M+3H]^{3+}$=530.0 $[M+2H]^{2+}$=795.0
4	Ac[d]	Trp	22.63/100	40.14/100	$[M+3H]^{3+}$=526.0 $[M+2H]^{2+}$=788.5	$[M+3H]^{3+}$=526.0 $[M+2H]^{2+}$=788.3
5	CH_3OCO	Phe	26.26/99	44.21/100	$[M+3H]^{3+}$=522.6 $[M+2H]^{2+}$=783.5 $[M+4H]^{4+}$=392.2	$[M+3H]^{3+}$=522.5 $[M+2H]^{2+}$=783.7 $[M+4H]^{4+}$=392.1
6	$C_6H_5OCH_2CO$	Phe	30.06/100	29.35/100[c]	$[M+4H]^{4+}$=411.0 $[M+3H]^{3+}$=547.7 $[M+5H]^{5+}$=329.0	$[M+4H]^{4+}$=411.0 $[M+3H]^{3+}$=547.7 $[M+5H]^{5+}$=329.0
7	Gly	Phe	24.92/100	37.28/100	$[M+4H]^{4+}$=391.7 $[M+3H]^{3+}$=522.0 $[M+5H]^{5+}$=313.6	$[M+4H]^{4+}$=391.7 $[M+3H]^{3+}$=521.9 $[M+5H]^{5+}$=313.6
8	Ac-Gly	Phe	22.66/100	40.75/100	$[M+4H]^{4+}$=402.2 $[M+3H]^{3+}$=536.0	$[M+4H]^{4+}$=402.2 $[M+3H]^{3+}$=536.0
9	CH_3OCO	Trp	26.33/100	43.61/100	$[M+3H]^{3+}$=535.3 $[M+2H]^{2+}$=802.5	$[M+3H]^{3+}$=535.3 $[M+2H]^{2+}$=802.4
10	$C_6H_5OCH_2CO$	Trp	29.34/99.6	28.03/97.7[c]	$[M+3H]^{3+}$=561.0 $[M+2H]^{2+}$=841.0	$[M+3H]^{3+}$=561.1 $[M+2H]^{2+}$=841.1
11	Gly	Trp	18.85/100	33.98/100	$[M+2H]^{2+}$=802.0 $[M+3H]^{3+}$=535.0	$[M+2H]^{2+}$=801.9 $[M+3H]^{3+}$=534.9
12	Ac-Gly	Trp	22.08/100	38.88/100	$[M+3H]^{3+}$=549.3 $[M+2H]^{2+}$=823.0	$[M+3H]^{3+}$=549.4 $[M+2H]^{2+}$=823.0
13	CH_3OCO	Tyr	24.32/100	41.00/100	$[M+3H]^{3+}$=527.6 $[M+2H]^{2+}$=791.0 $[M+4H]^{4+}$=396.2	$[M+3H]^{3+}$=527.7 $[M+2H]^{2+}$=791.1 $[M+4H]^{4+}$=396.3
14	CH_3OCO	Nal(2')	29.33/100	29.10/100[c]	$[M+3H]^{3+}$=539.3 $[M+4H]^{4+}$=404.5 $[M+2H]^{2+}$=808.0	$[M+3H]^{3+}$=539.4 $[M+4H]^{4+}$=404.5 $[M+2H]^{2+}$=808.1
15	CH_3OCO	Cha	29.33/100	29.10/100[c]	$[M+3H]^{3+}$=524.3 $[M+2H]^{2+}$=786.0 $[M+4H]^{4+}$=393.7	$[M+3H]^{3+}$=524.4 $[M+2H]^{2+}$=786.1 $[M+4H]^{4+}$=393.8
16	Ac	Tyr	22.83/100	38.41/100	$[M+3H]^{3+}$=523.0 $[M+2H]^{2+}$=784.0	$[M+3H]^{3+}$=522.9 $[M+2H]^{2+}$=783.8
17	Ac	Nal(2')	28.12/100	28.39/100[c]	$[M+3H]^{3+}$=534.0 $[M+4H]^{4+}$=400.7 $[M+2H]^{2+}$=800.5	$[M+3H]^{3+}$=533.9 $[M+4H]^{4+}$=400.7 $[M+2H]^{2+}$=800.4
18	Ac	Cha	27.27/100	27.78/100[c]	$[M+3H]^{3+}$=519.3 $[M+2H]^{2+}$=778.0 $[M+4H]^{4+}$=389.5	$[M+3H]^{3+}$=519.3 $[M+2H]^{2+}$=778.0 $[M+4H]^{4+}$=389.5

[a]System 1: Solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in acetonitrile. [b]System 2: Solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in methanol. The gradient for both systems was 5-50% solvent B over 45 min. [c]The gradient was 5-86% solvent B over 45 min. [d]Phe instead of NMePhe in position 1.

4.2.3 Pharmacology

The affinities of these peptides for rat κ and μ opioid receptors expressed on Chinese hamster ovary (CHO) cells were determined by competitive inhibition of the radioligands [^{3}H]diprenorphine and [^{3}H]DAMGO ([D-Ala^2,MePhe^4,glyol]-enkephalin), respectively (Tables 4.2 and 4.3).[28] Since all of the arodyn analogs reported so far show very low affinity for δ opioid receptors (i.e., K_i > 5 μM), only κ and μ opioid receptors affinities were evaluated.

[NMePhe^1]arodyn (**2**) was previously reported as the most potent and selective arodyn analog.[26] Substitution of the acetyl group in this compound with a methoxycarbonyl group resulted in compound **5** that has similar affinity for κ opioid receptors. The μ opioid receptor affinity increases by about 3-fold, however, and therefore, the selectivity for κ over μ opioid receptors decreases by 3-fold. Compared with **2**, a loss of κ opioid receptor affinity (2- to 8.7-fold) was observed when the N-terminal acetyl group of **2** was substituted with other acyl groups (compounds **6**-**8**). Substitution of the N-terminal acetyl group in **2** with the bulky phenoxylacetyl group (compound **6**) decreases κ opioid receptor affinity by 2-fold while increasing μ opioid receptors affinity by 3.5-fold; therefore, the selectivity of **6** for κ opioid receptors is 7-fold less than **2**. The largest decrease in κ opioid receptor affinity (8.7-fold) occurred when glycine is substituted for the acetyl group (compound **7**), likely as a result of the introduction of the positively charged amine group. This effect has also been observed for arodyn, in which the κ opioid receptor affinity decreased 4-fold upon removal of the N-terminal acetyl group.[26] The addition of this charged amine also increases the affinity for μ opioid receptors slightly so that this compound shows only 77-fold selectivity for κ over μ opioid receptors. Acetylation of the free amine (compound **8**) increases the affinity for κ opioid receptors 3-fold and decreases μ opioid receptor affinity (> 3.3-fold) compared to **7**, so that the resulting compound exhibits very high κ opioid receptor selectivity

(K_i ratio (κ/μ) = 1/>810). However, as compared to **2** and **5**, this compound shows about 2.5-fold lower κ opioid receptor affinity.

Table 4.2: Opioid receptor affinities and efficacies of arodyn (**1**) and its N-terminal substituted analogs

X-NMePhe-Phe-R_1-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2

#	X	R_1	K_i ± SEM (nM)		K_i ratio (κ/μ)	AC % inhibition @ 10 μM[a]
			κ	μ		
1, arodyn[b]	Ac[c]	Phe	10.0 ± 3.0	1750 ± 130	1/175	12 ± 8
2[d]	Ac	Phe	4.56 ± 0.45	5056 ± 790	1/1100	14 ± 1
5	CH_3OCO	Phe	4.93 ± 0.30	1750 ± 97	1/335	6 ± 10 (4)
6	$C_6H_5OCH_2CO$	Phe	9.32 ± 0.44	1460 ± 190	1/157	15 ± 25
7	Gly	Phe	39.8 ± 5.1	3070 ± 130	1/77	24 ± 9 (2)
8	Ac-Gly	Phe	12.3 ± 3.2	>10,000	1/>810	37 ± 5 (4)
3	Ac	Trp	13.0 ± 2.6	2020 ± 560	1/155	24 ± 9
4	Ac[c]	Trp	16.1 ± 2.1 (4)	1180 ± 290	1/73	-3 ± 1 (4)
9	CH_3OCO	Trp	9.69 ± 1.82	1280 ± 280	1/132	8 ± 12 (5)
10	$C_6H_5OCH_2CO$	Trp	13.3 ± 3.6 (4)	1250 ± 140	1/94	ND[e]
11	Gly	Trp	15.4 ± 2.1	500 ± 150 (4)	1/32	3 ± 5
12	Ac-Gly	Trp	21.9 ± 4.8	2910 ± 470	1/133	10 ± 3

[a]Compared to Dyn A (1-13)NH_2 (100%). [b]From reference 24. [c]Phe instead of NMePhe in position 1. [d]From reference 26. The values are the mean ± SEM for three independent experiments except where noted. [e]Not determined.

[NMePhe[1],Trp[3]]arodyn (**3**) shows similar affinity and selectivity (Table 4.2) for κ opioid receptors as arodyn (**1**). However, this analog has about 3-fold lower affinity than [NMePhe[1]]arodyn (**2**), suggesting that a larger aromatic group in position 3 is detrimental to κ opioid receptor affinity. Compared with **3**, its Phe[1] analog **4** shows similar affinity (Table 4.2) for κ opioid receptors; however, this compound exhibits about 2-fold higher affinity for μ opioid receptors, resulting in 2-fold lower selectivity for κ opioid receptors than compound **3**. These results once again indicate that the N-methyl group on Phe[1] increases κ opioid receptor selectivity, probably due to the effect of methylation on orientation of the acetyl group and/or side chain conformation. When the N-terminal acetyl group is replaced by a methoxycarbonyl group (compound **9**), the affinity at κ opioid receptors is similar, while the

affinity at μ opioid receptors increases by 2-fold, resulting in a very slight decrease in κ opioid receptor selectivity compared to **3**. Substitution of the N-terminal acetyl group with the larger phenoxylacetyl group (compound **10**) does not affect κ opioid receptor affinity, but also results in a slight increase in affinity for μ opioid receptors; therefore, the selectivity at κ opioid receptors is slightly lower than **3**. The largest increase in μ opioid receptor affinity occurred when glycine is substituted for the acetyl group (compound **11**), most likely as a result of the introduction of the positively charged amine group, resulting in a compound with only modest selectivity (32-fold) for κ opioid receptors. Acetylation of the free amine in this peptide gives compound **12** with decreased μ opioid receptor affinity so that **12** exhibits high κ opioid receptor selectivity. However, as compared with the parent peptide **3**, this compound shows about a 2-fold decrease in κ opioid receptor affinity.

The effects of the substitution in the two series of compounds (Phe^3 and Trp^3) are somewhat different. For example, in the [$NMePhe^1$]arodyn analogs, incorporating substitutents other than a methoxycarbonyl group results in lower affinity for κ opioid receptors. But this is not true for most of the [$NMePhe^1$,Trp^3]arodyn analogs. Compound **7** shows 8.7-fold lower affinity for κ opioid receptor than its methoxycarbonyl analog **5**, while compound **11** has almost the same affinity as its methoxycarbonyl analog **9**. The substitution also has different effects on μ opioid receptor affinity. Compound **7** shows about 2-fold lower affinity for μ opioid receptors than its methoxycarbonyl analog **5**, while compound **11** shows 2.5-fold higher affinity for μ opioid receptor than as its methoxycarbonyl analog **9**. These results suggest that the residue in 3 affects the orientation of the N-terminal substitutent and/or N-methyl group in the binding pocket, which in turn affects the pharmacological activity.

Thus the acetyl group in the parent compounds can best be substituted with a methoxycarbonyl group to maintain affinity and selectivity for κ opioid receptors. The

methoxycarbonyl group has similar size and other physicochemical properties as the acetyl group, and therefore the biological activity is preserved.

We then prepared stable N-terminal methyloxylcarbonyl analogs for comparison to the corresponding unstable acetylated peptides with various amino acids in position 3 (Table 4.3). Two of the methoxycarbonyl analogs, [CH_3OCO-NMePhe^1,Tyr^3]arodyn (**13**) and [CH_3OCO-NMePhe^1,Cha^3]arodyn (**15**), retain similar affinity for κ opioid receptors as [CH_3OCO-NMePhe^1]arodyn (**5**), suggesting that substituting of Phe in position 3 with a potential H-donor or acceptor amino acid Tyr or saturated analog Cha is tolerated by κ opioid receptors. [CH_3OCO-NMePhe^1,Nal(2')^3]arodyn (**14**), with substitution of Phe with a bulky aromatic amino acid Nal(2), however, shows about a 5-fold loss in affinity for κ opioid receptors compared to **5**. This result is similar to the other analog with a bulky aromatic substitution in that position, [CH_3OCO-NMePhe^1,Trp^3]arodyn (**9**). Interestingly, even though [CH_3OCO-NMePhe^1,Tyr^3]arodyn (**13**) and [CH_3OCO-NMePhe^1,Cha^3]arodyn (**15**) have similar affinity for κ opioid receptors as [CH_3OCO-NMePhe^1]arodyn (**5**), this is not the case for their corresponding methoxycarbonyl analogs (Table 4.3). [MePhe^1,Tyr^3]arodyn (**16**) and [MePhe^1,Cha^3]arodyn (**18**) have lower affinity (6- and 3-fold, respectively) for κ opioid receptors than their corresponding N-terminal methoxycarbonyl analogs **13** and **15**. [MePhe^1,Nal(2')^3]arodyn (**17**), however, has almost the same κ opioid receptor affinity as its methoxycarbonyl analog **14**. These results once again suggest that the residue in 3 affects the orientation of the N-terminal substitutent in the binding pocket, which in turn affects the pharmacological activity.

Table 4.3: Opioid receptor affinity and efficacy of [NMePhe[1]]arodyn analogs where position 3 was substituted with other aromatic and non-aromatic amino acids

X-NMePhe-Phe-R_1-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2

#	X	R_1	$K_i \pm$ SEM (nM)		K_i ratio (κ/μ)	AC % inhibition @ 10 μM[a]
			κ	μ		
5	CH_3OCO	Phe	4.93 ± 0.30	1750 ± 97	1/335	6 ± 10 (4)
9	CH_3OCO	Trp	9.69 ± 1.82	1280 ± 280	1/132	8 ± 12 (5)
13	CH_3OCO	Tyr	5.80 ± 1.27	1630 ± 120	1/271	35 ± 11
14	CH_3OCO	Nal(2')	24.6 ± 7.2 (6)	51.3 ± 12.7 (4)	1/2	ND[b]
15	CH_3OCO	Cha	3.60 ± 0.21	863 ±352	1/240	18 ± 10
2[c]	Ac	Phe	4.56 ± 0.45	5056 ± 790	1/1100	14 ± 1
3	Ac	Trp	13.0 ± 2.6	2020 ± 560	1/155	24 ± 9
16[d]	Ac	Tyr	37.3 ± 16.0	3370 ± 570 (5)	1/90	9 ± 8
17[d]	Ac	Nal(2')	27.0 ± 7.3	158 ± 50	1/6	37 ± 4
18[d]	Ac	Cha	10.3 ± 1.8	NA	ND[b]	43 ± 4

[a]Compared to Dyn A (1-13)NH_2 (100%). [b]Not determined. [c]From reference 26. [d]Bennett, Murray and Aldrich, unpublished results.

The κ receptor selectivity of these methoxycarbonyl analogs is not as high as [CH_3OCO-MePhe[1]]arodyn, **5** (Table 4.3). [CH_3OCO-MePhe[1],Nal(2')[3]]arodyn (**14**) shows much higher μ opioid receptor affinity compared to the other analogs, and therefore this compound exhibits only 2-fold selectivity for κ over μ opioid receptors. Its acetylated analog **17** also has high affinity for μ opioid receptors (Table 4.3). Unlike other arodyn analogs, these two compounds are exceptions that they exhibit significant affinity for μ opioid receptors. The bulky hydrophobic aromatic side chain in position 3 appears to be responsible for the increased affinity for μ opioid receptors.

These arodyn analogs were evaluated for their intrinsic activity at 10 μM in the adenylyl cyclase (AC) assay using cloned rat κ opioid receptors stably expressed in CHO cells.[29] Most of the analogs exhibit negligible or very low efficacy, similar to arodyn (≤ 25% of the maximum inhibition of AC relative to Dyn A-(1-13)NH_2, Tables 4.2 and 4.3). Only

four of the compounds tested to date, **8, 13**, **17**, and **18**, exhibited > 25% of the maximum inhibition of AC relative to Dyn A-(1-13)NH_2.

[CH_3OCO-NMePhe^1]arodyn (**5**) was evaluated for its antagonist activity by reversing the agonism of Dyn A-(1-13)NH_2 in the AC assay using CHO cells stably expressing κ opioid receptors. The K_B value was 2.6 ± 1.7 nM (n = 2), indicating that it is a κ opioid receptor antagonist.

4.3 Conclusions

[NMePhe^1]arodyn (**2**) exhibits high affinity and selectivity for κ opioid receptors. However, this peptide is unstable and prone to deletion of the Ac-NMePhe moiety under acidic conditions. By replacing the acetyl group with heteroatom-containing groups, stable analogs can be obtained. Pharmacological evaluation showed that a methoxycarbonyl group could be substituted for the acetyl group in [NMePhe^1]arodyn without loss of κ opioid receptor affinity. [CH_3OCO-NMePhe^1]arodyn (**5**) has similar κ opioid receptor affinity as [NMePhe^1]arodyn (**2**) and retains high κ opioid receptor selectivity. For other substituents (i.e. $C_6H_5OCH_2CO$, Gly, and Ac-Gly), the affinity and/or selectivity for κ receptors decreases compared to the parent peptide **2**. In the [NMePhe^1,Trp^3]arodyn series of analogs, substitutents other than CH_3OCO have similar effects on κ opioid receptor affinity, except for Ac-Gly which decreases the κ opioid receptor affinity 2-fold. However, the selectivity for κ over μ opioid receptors is generally not as high as when CH_3OCO was the N-terminal substitutent.

[NMePhe^1]arodyn (**2**) has higher affinity than other analogs where Phe^3 was replaced with other aromatic or non-aromatic amino acids. However, the N-terminal methoxycarbonyl analogs where Phe^3 was replaced with other aromatic or non-aromatic amino acids generally retain similar affinity for κ opioid receptors as [CH_3OCO-NMePhe^1]arodyn (**5**). The

selectivity of these methoxycarbonyl analogs for κ over μ opioid receptors, however, was generally lower than **5**.

The analogs described in this chapter generally exhibit negligible or very low efficacy in the AC assay, similar to arodyn. [CH_3OCO-NMePhe^1]arodyn (**5**) reverses the agonism of Dyn A-(1-13)NH_2 at κ opioid receptors in the AC assay with high potency (K_B = 2.6 nM). Thus we have successfully identified highly selective, potent, and stable [NMePhe^1]arodyn analogs for κ opioid receptors. [CH_3OCO-NMePhe^1]arodyn could prove to be quite useful as a pharmacological tool to compliment the non-peptide κ receptor selective antagonists to study the physiological processes mediated by κ opioid receptors and also as a lead peptide for further modification.

4.4 Experimental section

4.4.1 Materials

Fmoc-Nal(2')-OH was purchased from Peptides International (Louisville, KY). Fmoc-Cha-OH was purchased from Calbiochem-Novabiochem (San Diego, CA). The sources of other materials are listed in Chapter 3.

4.4.2 Peptide synthesis and cleavage

Peptide Synthesis: All peptides were synthesized on the Fmoc-PAL-PEG-PS resin (0.16-0.24 mmol/g) by standard SPPS methods (for more details, please refer to Chapter 3). For the cleavage of the stable analogs, Reagent B (85% TFA, 5% phenol, 5% water, and 2% triisopropylsilane)[27] at room temperature for 2 h was used. For the cleavage of unstable analogs (i.e. **2**, **3**, and **16-18**), pure TFA without any scavenger at 4 ^oC for 3 h was used (see Chapter 3).

4.4.3 Purification and analysis of peptides

The crude peptides were purified by preparative reversed-phase HPLC (Shimadzu HPLC system equipped with a Shimadzu SPD-10A detector) on a Vydac C18 column (10 μ, 300 Å, 21 x 250 mm) equipped with a Vydac guard cartridge. For purification, a linear gradient of 15-50% MeCN containing 0.1% TFA over 35 min, at a flow rate of 20 mL/min, was used. The purification was monitored by UV absorbance at 214 nm.

The purity of the final peptides was verified using analytical HPLC in two different solvent systems using a Shimadzu HPLC system equipped with a Shimadzu SPD-10A detector and a Vydac C18 column (5 μ, 300 Å, 4.6 x 50 mm) equipped with a Vydac guard cartridge. In system 1, a linear gradient of 5-50% solvent B (solvent A, aqueous 0.1% TFA, and solvent B, MeCN containing 0.1% TFA) over 45 min, at a flow rate of 1 mL/min, was used with monitoring at 214 nm. In system 2, a linear gradient of 5-50% solvent B (solvent A, aqueous 0.1% TFA, and solvent B, MeOH containing 0.1% TFA) over 45 min, at a flow rate of 1 mL/min, was used with monitoring at 230 nm. However, for the more hydrophobic peptides (i.e. **6**, **10**, **14**, **15**, **17**, and **18**), a gradient of 5-86% solvent B (methanol containing 0.1% TFA) over 45 min was used for the analysis. The final purity of all peptides in both analytical systems was generally greater than 98% (Table 4.1). Interestingly, the retention times for the methoxycarbonyl analogs were longer than for the corresponding acetyl analogs. The molecular weights of the compounds were determined by ESI-MS.

4.4.4 Radioligand binding assays

Radioligand binding assays were performed as previously described using cloned rat κ and μ opioid receptors stably expressed on CHO cells.[28] [^{3}H]Diprenorphine and [^{3}H]DAMGO ([D-Ala^2,MePhe^4,glyol]enkephalin) were used as radioligands in the assays for κ and μ receptors, respectively. Nonspecific binding was determined in the presence of 10

μM unlabeled Dyn A-(1-13)NH_2 and DAMGO for κ and μ receptors, respectively. Binding assays were carried out under standard conditions in the presence of peptidase inhibitors (10 μM bestatin, 30 μM captopril, and 50 μM L-leucyl-L-leucine) and 3 mM Mg^{2+}. IC_{50} values were determined by nonlinear regression analysis to fit a logistic equation to the competition data using GraphPad Prism software (GraphPad Software Co., San Diego, CA). K_i values were calculated from the IC_{50} values by the Cheng and Prusoff equation,[30] using K_D values of 0.45 and 0.49 nM for [^{3}H]diprenorphine and [^{3}H]DAMGO, respectively. The results presented (Tables 4.2 and 4.3) are the mean ± SEM from three separate assays except where otherwise noted.

4.4.5 Adenylyl cyclase assays

The AC assay was performed using cloned rat κ opioid receptors stably expressed on CHO cells as previously described.[29] Cell cultures were washed twice with free F12 medium and then incubated for 4 hours in 1 mL of the same media containing 12μCi [^{3}H]adenine. The media was incubated at 37 ^oC for 40 min in the presence of 50 mM forskolin, peptidase inhibitors (10 μM bestatin, 30 μM captopril, and 50 μM L-leucyl-L-leucine) and 10 μM the peptide ligand. Incubations were terminated by the addition of 30 μL of Stop solution (2% sodium dodecyl sulfate and 1.3 mM cyclic adenosine monophosphate (cAMP) in water), followed by the addition of 100 μL of conc perchloric acid and 750 μL water. [^{14}C]cAMP (500 cpm in 50 μL) was added to each well to correct for recovery. After transferring the contents of the wells to 1.5 mL centrifuge tubes, 12 M KOH was added to neutralize the samples. The resulting precipitates were pelleted by centrifugation at 10,000 *g* for 10 minutes. cAMP in the supernatants was isolated by sequential chromatography over BioRad AG-50W-X4 cation exchange resin and neutral alumina. The concentrations of [^{3}H]cAMP

and [^{14}C]cAMP in the eluants were determined simultaneously by scintillation counting. Counts were corrected for crossover and recovery.

4.5 Bibliography

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Chapter 5. Identification and Structure-Activity Relationships of Arodyn Analogs Exhibiting Inverse Agonist and Agonist Activity at Kappa Opioid Receptors

*Note that the compound numbers used in this chapter are only applicable within this specific chapter.

5.1 Introduction

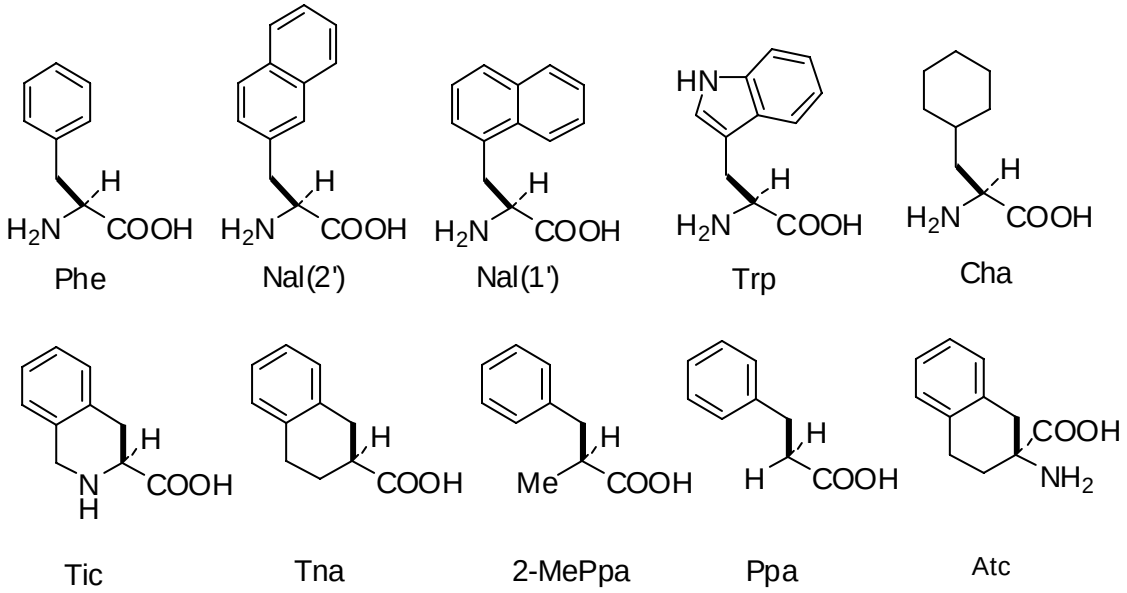
Our research focuses on the development of potent and selective kappa (κ) opioid receptor ligands as pharmacological tools and potential therapeutics. For a general overview of κ opioid receptors, κ opioid receptor ligands and their potential therapeutic applications, please refer to previous chapters.

Opioid receptors belong to the superfamily of G-protein coupled receptors (GPCR).[1] In this family of receptors, the signal transduction is achieved by the agonist occupied receptors activating one or more G-proteins. Activation of opioid receptors mediates inhibition of adenylyl cyclase (AC) activity, which results in decreased production of cyclic adenosine monophosphate (cAMP). On the other hand, opioid receptor inverse agonists stimulate AC activity. According to the two-state receptor theory, agonists and inverse agonists are thought to have selective binding affinity for the pre-existing active and resting states.[2, 3] Therefore, identification of novel inverse agonists for opioid receptors may aid in the understanding of how ligand-receptor interactions can shift the receptor states. Reports of opioid receptor ligands with inverse agonist activity have been limited.[4-10] Statnick and coworkers have shown that compounds with delta (δ) receptor inverse agonist activity may be useful as anorectants.[7, 8]

Arodyn (Ac[Phe^{1,2,3},Arg^{4},D-Ala^{8}]Dyn A(1-11)-NH_2, Ac-Phe-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2, **1**) is an acetylated dynorphin A (Dyn A) analog that is a potent and selective κ opioid receptor antagonist.[11] Structure-activity relationship (SAR) studies of arodyn showed that the Phe residues at positions 1 and 3 in the "message"[12] sequence contribute to the κ opioid receptor affinity of arodyn (Ala

substitution of either of these two residues resulted in a 3- to 4-fold loss in affinity),[10] but it was not known whether Phe was the optimum residue at these positions. Therefore a combinatorial library of arodyn analogs was constructed in which the amino acids in positions 1 and 3 were varied.[13] Amino acids incorporated included Phe, Trp, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic), 2-naphthylalanine (Nal(2')), and cyclohexylalanine (Cha) (Figure 5.1). Peptides were chosen for further evaluation based on their κ opioid receptor affinity.

Figure 5.1: Structures of Phe, Nal(2'), Nal(1'), Trp, Cha, Tic, Tna, 2-MePpa, Ppa, and Atc



Arodyn and most of its analogs have negligible efficacy at κ opioid receptors. However, two analogs in the library exhibited inverse agonist activity at κ opioid receptors. [Nal(2')^{1,3}]arodyn (**2**) shows high affinity for κ opioid receptors and stimulates AC activity (maximum 38% stimulation over untreated controls compared to 100% inhibition by Dyn A, Table 5.2).[13] [Nal(2')^{1},Trp^{3}]arodyn (**3**) also stimulates AC activity

(maximum 28% stimulation over untreated controls, Table 5.2).[13] Most of the reported opioid receptor inverse agonists have selectivity for δ opioid receptors.[4-9] The only other Dyn A analog reported to exhibit selective inverse agonist activity at κ opioid receptors is also an arodyn analog ([Tyr^1]arodyn) identified in our laboratory.[10] Therefore we explored the structural requirements for the inverse agonist activity of these analogs. We evaluated the effects of substitution of residues 1 and 3 on κ opioid receptor affinity, selectivity, and efficacy.

On the other hand, two analogs from this library exhibit agonist activity at κ opioid receptors.[13] [Tic^1,Cha^3]arodyn (**4**) shows almost full agonist activity at κ opioid receptors (Table 5.3), as does its analog where Arg^4 is substituted with Lys (compound **5**).[13] These are the only two arodyn analogs that show nearly full agonist activity. Therefore it was a high priority to prepare additional analogs to assess the structural requirements for agonist *vs* antagonist activity. Our hypothesis was that changes in residue 3 (from Phe to Cha) may shift the position of Tic^1 in the binding site such that hydrogen bonding of the N-terminal moiety with a group on the receptor changes the receptor conformation and results in receptor activation. Acetylated Tic could act as a hydrogen bond acceptor through the carbonyl oxygen of the acetyl group. Eliminating the carbonyl group would eliminate the H-bonding capability, and therefore could prevent receptor activation by the Cha^3 peptides. Therefore we prepared arodyn analogs containing 1,2,3,4-tetrahydro-2-naphthoic acid (Tna), phenylproprionic acid (Ppa) and its 2-methyl derivative (2-MePpa, Figure 5.1) in place of Ac-Tic^1. Also analogs containing Phe^3 were compared to the Cha^3 analogs to assess whether H-bonding capability is important for antagonist activity for κ opioid receptors.

Therefore, the primary object of this part of the research was to study the SAR of arodyn analogs exhibiting inverse agonist and agonist activity. This report will be divided into two sections. The first section focuses on the SAR of arodyn analogs exhibiting inverse agonist activity, while the second section focuses on the SAR of arodyn analogs exhibiting agonist activity. These studies could assist in our understanding of arodyn-κ opioid receptor interactions and how subtle structural changes can influence κ opioid receptor affinity, selectivity, and especially efficacy.

5.2 Results and discussion

5.2.1 Chemistry

The peptides were synthesized on the Fmoc-PAL-PEG-PS resin (Fmoc: 9-fluorenylmethoxycarbonyl; PAL-PEG-PS: peptide amide linker-poly(ethylene glycol)-polystyrene, 0.19 mmol/g) by solid-phase peptide synthesis using Fmoc-protected amino acids following standard procedures (see Chapter 3 for details). The syntheses were performed using benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBt), and *N*,*N*-diisopropylethylamine (DIEA) in DMF as the coupling reagents. After assembling the protected peptides on the resin, these peptides were cleaved with Reagent B (88% trifluoroacetic acid (TFA), 5% phenol, 5% water, and 2% triisopropylsilane)[14] and purified by reversed-phase high performance liquid chromatography (RP-HPLC). The purity of the purified peptides was greater than 98% as verified by two different HPLC solvent systems (MeCN/water and MeOH/water, both containing 0.1% TFA). The molecular weight of these peptides was verified by

electrospray ionization mass spectrometry (ESI-MS) analysis. The analytical data for the peptides is presented in Table 5.1.

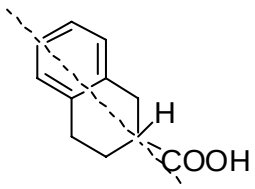
Table 5.1: Analytical data for purified arodyn analogs substituted in positions 1 and 3

Peptides (Modifications to arodyn)	HPLC (t_R (min)/% Purity)		ESI-MS (*m/z*)	
	System 1[a]	System 2[b]	Calculated	Observed
6 [Nal(2')^3]	26.38/100	26.63/100[c]	$[M+3H]^{3+}$=529.3 $[M+2H]^{2+}$=793.0 $[M+4H]^{4+}$=397.2	$[M+3H]^{3+}$=529.3 $[M+2H]^{2+}$=792.9 $[M+4H]^{4+}$=397.2
7 [Nal(1')^3]	25.56/100	43.80/100	$[M+3H]^{3+}$=529.0 $[M+2H]^{2+}$=793.5 $[M+4H]^{4+}$=397.0	$[M+3H]^{3+}$=528.9 $[M+2H]^{2+}$=793.4 $[M+4H]^{4+}$=396.9
8 [Trp^3]	22.63/100	40.14/100	$[M+3H]^{3+}$=526.0 $[M+2H]^{2+}$=788.5	$[M+3H]^{3+}$=526.0 $[M+2H]^{2+}$=788.3
9 [Nal(2')^1]	25.51/100	25.99/98.5[c]	$[M+4H]^{4+}$=397.0 $[M+3H]^{3+}$=529.0	$[M+4H]^{4+}$=397.0 $[M+3H]^{3+}$=529.0
10 [Nal(2')^1,Tyr^3]	24.27/100	25.48/100[c]	$[M+3H]^{3+}$=534.3 $[M+2H]^{2+}$=801.0	$[M+3H]^{3+}$=534.3 $[M+2H]^{2+}$=800.9
12 [Cha^3]	26.06/100	26.82/100[c]	$[M+3H]^{3+}$=514.3 $[M+4H]^{4+}$=386.0 $[M+2H]^{2+}$=771.0	$[M+3H]^{3+}$=514.3 $[M+4H]^{4+}$=386.0 $[M+2H]^{2+}$=771.0
13 [D-Tic^1,Cha^3]	26.45/100	26.61/100[c]	$[M+3H]^{3+}$=518.3 $[M+4H]^{4+}$=389.0 $[M+2H]^{2+}$=777.0	$[M+3H]^{3+}$=518.3 $[M+4H]^{4+}$=389.0 $[M+2H]^{2+}$=777.0
14 [Ppa^1,Cha^3]	28.15/100	27.82/100[c]	$[M+3H]^{3+}$=495.3 $[M+4H]^{4+}$=371.7 $[M+2H]^{2+}$=742.5	$[M+3H]^{3+}$=495.3 $[M+4H]^{4+}$=371.7 $[M+2H]^{2+}$=742.5
15 [(S/R)-2-MePpa^1,Cha^3]	28.32/100	28.82/100[c]	$[M+3H]^{3+}$=500.3 $[M+2H]^{2+}$=750.0 $[M+4H]^{4+}$=375.5	$[M+3H]^{3+}$=500.3 $[M+2H]^{2+}$=750.0 $[M+4H]^{4+}$=375.5
16 [(S/R)-Tna^1, Cha^3]	30.09/100	29.62/100[c]	$[M+3H]^{3+}$=504.3 $[M+2H]^{2+}$=756.0 $[M+4H]^{4+}$=378.5	$[M+3H]^{3+}$=504.3 $[M+2H]^{2+}$=756.0 $[M+4H]^{4+}$=378.7
17 [Ppa^1]	26.07/99.4	42.84/100	$[M+3H]^{3+}$=493.6 $[M+4H]^{4+}$=370.2 $[M+2H]^{2+}$=739.4	$[M+3H]^{3+}$=493.6 $[M+4H]^{4+}$=370.2 $[M+2H]^{2+}$=739.5
18 [(S/R)-2-MePpa^1]	26.96/100	27.71/100[c]	$[M+3H]^{3+}$=498.0 $[M+4H]^{4+}$=373.7 $[M+2H]^{2+}$=746.5	$[M+3H]^{3+}$=497.9 $[M+4H]^{4+}$=373.7 $[M+2H]^{2+}$=746.5
19 [(S/R)-Tna^1]	27.54/98.0	27.28/100[c]	$[M+3H]^{3+}$=502.0 $[M+2H]^{2+}$=752.5 $[M+4H]^{4+}$=377.0	$[M+3H]^{3+}$=502.2 $[M+2H]^{2+}$=752.5 $[M+4H]^{4+}$=377.0

[a]System 1: Solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in acetonitrile. [b]System 2: Solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in methanol. The gradient for both systems was 5-50% solvent B over 45 min. [c]The gradient was 5-86% solvent B over 45 min.

Initially, the peptides containing 2-MePpa and Tna were synthesized using the racemic acids. The resulting diastereomeric peptides were not separable by HPLC. Because of the high κ receptor affinity (see below), resolution of the enantiomers of these acids was attempted. Previously our laboratory reported that the structurally related racemic 2-aminotetralin-2-carboxylic acid (Atc, Figure 5.1) can be resolved by converting to diastereomeric tripeptides Bz-(R,S)-Atc-(S)-Phe-(S)-PheN$(CH_2)_4$ followed by fractional crystallization.[15] Based on this observation, we successfully resolved 2-MePpa by coupling to Phe-NH_2. The two diastereomers were then separated by HPLC using the MeOH/water system (see below for details). These two diastereomers were hydrolyzed by 3 M HCl and the pure enantiomers of 2-MePpa isolated. The corresponding diastereomeric mixture of Tna-Phe-NH_2, however, could not be separated. The Tna isomers also could not be separated by preparing other diastereomeric amino acid (Pro-NH_2) or dipeptide (Pro-Lys-NH_2) derivatives, presumably due to the pseudo-symmetrical structure of the acid (Figure 5.2). Other approaches will need to be explored to resolve the isomers of this acid.

Figure 5.2: Pseudo-symmetrical structure of Tna



Tna

5.2.2 SAR of arodyn analogs exhibiting inverse agonist activity

The affinity of these peptides for rat κ and mu (μ) opioid receptors expressed on Chinese hamster ovary (CHO) cells was determined by competitive inhibition of the radioligands [^{3}H]diprenorphine and [^{3}H]DAMGO ([D-Ala^2,MePhe^4,glyol]-enkephalin), respectively (Tables 5.2 and 5.3).[16] Since all of the arodyn analogs reported to date show very low affinity for δ opioid receptors (K_i > 5 μM), only κ and μ opioid receptor affinities were evaluated.

The peptides were initially screened at 10 μM for their ability to inhibit or stimulate the synthesis of cAMP by AC under standard assay conditions (50 μM forskolin, Tables 5.2 and 5.3).[17] Several analogs showed inverse agonist activity in the initial screening assays. Selected analogs were examined for dose-dependent stimulation or inhibition of AC activity.

Compounds **2**, **3** and their analogs **6-10** were examined for opioid receptor affinity and inverse agonist activity. Compound **2**, with substitution of Nal(2') in both positions 1 and 3, shows a 2-fold increase in κ opioid receptor affinity compared with arodyn (Table 5.2). However, the affinity of this compound for μ opioid receptors also increases by almost 3-fold, resulting in slightly lower selectivity than arodyn. Compound **3**, with Nal(2') in position 1 and Trp in position 3, retains similar affinity for κ opioid receptors as arodyn while exhibiting the highest affinity (K_i = 327 nM) for μ opioid receptors, resulting in only modest (25-fold) selectivity for κ over μ opioid receptors (Table 5.2). These two compounds show inverse agonist activity in the initial screening assays.

Table 5.2: Pharmacological activities of [Nal(2')^{1,3}]arodyn (**2**) and its analogs

Arodyn (**1**): Ac-Phe-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2

Peptides (modifications to arodyn)	K_i ± SEM (nM)[a]		K_i ratio(κ/μ	AC % inhibition @ 10 μM[a,b]
	κ	μ		
1, arodyn[c]	10.0 ± 3.0	1740 ± 130	1/175	12 ± 8
2 [Nal(2')^{1,3}][d]	5.44 ± 1.0 (4)	587 ± 51	1/108	-38 ± 11[e]
3 [Nal(2')^1,Trp^3][d]	12.9 ± 1.4 (4)	327 ± 89 (4)	1/25	-28 ± 4[e]
6 [Nal(2')^3]	12.0 ± 1.5 (4)	1370 ± 280	1/114	-34 ± 8 (4)[e]
7 [Nal(1')^3]	12.9 ± 3.1 (6)	1530 ± 360	1/119	-39 ± 6 (4)[e]
8 [Trp^3]	16.1 ± 2.1 (4)	1180 ± 290	1/73	-7 ± 4 (4)[e]
9 [Nal(2')^1]	60.3 ± 12.1	1020 ± 370 (4)	1/17	25 ± 21
10 [Nal(2')^1,Tyr^3]	31.6 ± 0.8	6130 ± 2190	1/194	4.1 (1)

[a]The results are expressed as the mean ± SEM % of 3 independent experiments except where noted. [b]Compared to Dyn A (1-13)NH_2 (100%). [c]Data from reference 11. [d]Data from reference 13. [e]A negative number indicates stimulation of AC.

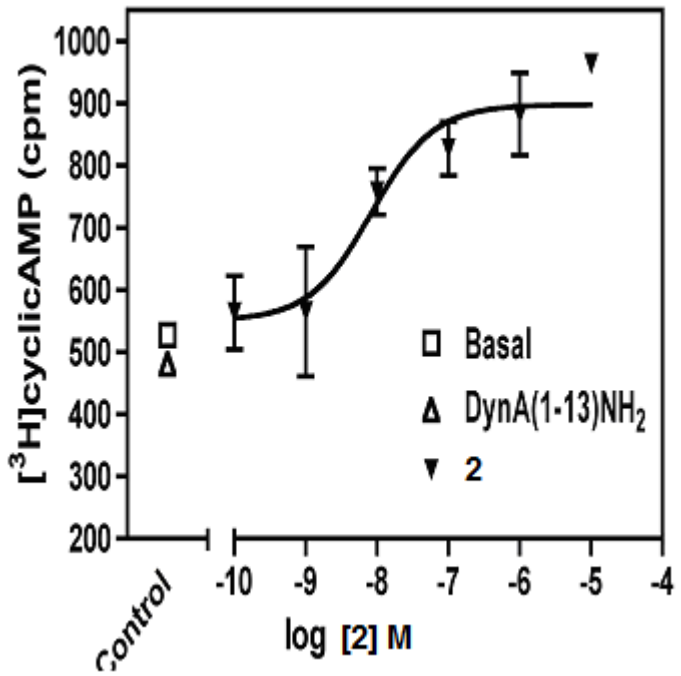
A bulky amino acid in position 3 in the arodyn analogs is generally tolerated by κ opioid receptors while slightly increasing affinity for μ opioid receptors compared with arodyn, therefore resulting in decreased selectivity for κ over μ opioid receptors. Substitution of Nal(2') in position 3 of arodyn results in a compound (**6**) with similar affinity for κ opioid receptors and slightly increased affinity for μ opioid receptors, and consequently a small loss in selectivity for κ over μ opioid receptors (Table 5.2). Substitution of Nal(1') in that position results in a compound (**7**) with an almost identical binding profile for κ and μ opioid receptors as **6** (Table 5.2). Substitution of Trp in position 3 (compound **8**) results in somewhat decreased affinity for κ opioid receptors and somewhat increased affinity for μ opioid receptors, and therefore about 2-fold lower selectivity for κ over μ opioid receptors. The residue at position 3 appears to be important for the observed inverse agonist activity. Compounds **2**, **3**, **6**, and **7** where position 3 was substituted with a bulky aromatic amino acid (Nal(1'/2') or Trp) show inverse agonist

activity (28-39% maximum stimulation of AC activity, Table 5.2). Compound **8** is an exception; even though position 3 is also a bulky aromatic amino acid (Trp), it shows negligible stimulation or inhibition of AC activity (Table 5.2).

Whether or not a bulky amino acid in position 1 is tolerated by κ opioid receptors appears to depend on the residue in position 3. [Nal(2')^1]arodyn (**9**), containing Phe in position 3, and [Nal(2')^1,Tyr^3]arodyn (**10**) show 3- and 6-fold lower affinity for κ opioid receptors, respectively, compared to arodyn (Table 5.2). Compound **9** also shows decreased selectivity (10-fold) for κ opioid receptors compared to arodyn (**1**). However, substitution of Nal(2') or Trp in position 3 (as in **2** and **3**) can restore the affinity for κ opioid receptors. Compounds **9** and **10** as well as arodyn (**1**) with Phe or Tyr in position 3 do not show inverse agonist activity, suggesting that the residue in position 1 is not important for inverse agonist activity.

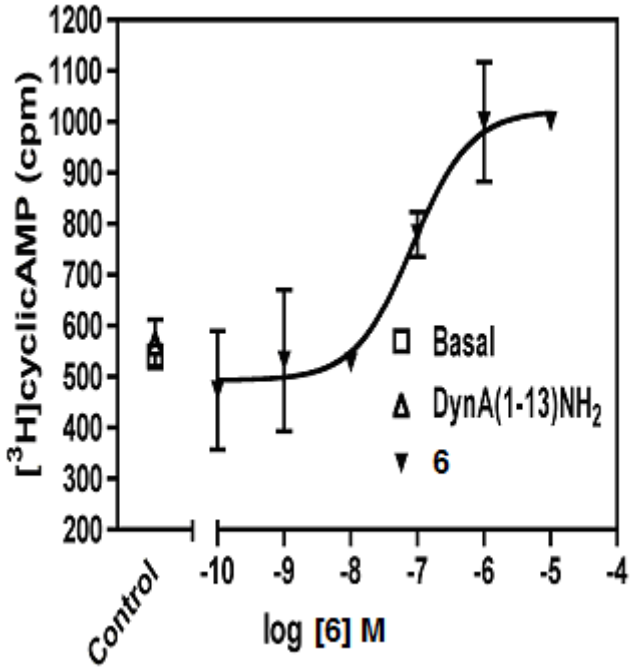
Analogs **2** and **6** were then examined for dose-dependent stimulation of AC activity under basal conditions (without added forskolin). Under these conditions, these two compounds stimulate the AC activity compared to basal levels. Both **2** and **6** exhibit dose-dependent inverse agonist activity (EC_{50} = 84 and 9.0 nM, respectively, in the initial assays, Figure 5.3).

Figure 5.3: Dose-dependent inverse agonist activity of **2** (a) and **6** (b) under basal conditions



(a)

(b)



5.2.3 SAR of arodyn analogs exhibiting agonist activity

[Tic[1],Cha[3]]arodyn (**4**) and its analogs **5**, and **11-19** were examined for opioid receptor affinity and for their efficacy at κ opioid receptors. Compound [Tic[1],Cha[3]]arodyn (**4**) has a 3.5-fold increase in affinity for κ opioid receptors compared to arodyn (Table 5.3).[13] However, its κ/μ selectivity is 2-fold lower than arodyn due to an approximately 7-fold increase in affinity for μ opioid receptors. Its Lys[4] analog [Tic[1],Cha[3],Lys[4]]arodyn (**5**) exhibits sub-nanomolar (K_i = 0.95 nM) binding affinity for κ opioid receptors and greater than 10-fold higher affinity for κ opioid receptors compared to arodyn.[13] This compound (**5**) also exhibits slightly higher selectivity (272-fold) for κ over μ opioid receptors compared to arodyn. These two compounds (**4** and **5**) exhibit almost full agonist activity at κ opioid receptors with a maximum of 83% and 80% AC inhibition, respectively, compared to the full agonist Dyn A (1-13)NH_2 (100% inhibition, Table 5.3).[13]

The respective importance of Tic[1] and Cha[3] residues was evaluated by examining [Tic[1]]- and [Cha[3]]arodyn (**11** and **12**, respectively, Table 5.3). Substitution of Phe in position 1 of arodyn with Tic (compound **11**) is well tolerated by κ opioid receptors while slightly increasing affinity (about 2-fold) for μ opioid receptors compared with arodyn, therefore resulting in slightly decreased selectivity for κ over μ opioid receptors. Substitution of Phe in position 3 of arodyn with Cha (compound **12**) is also well tolerated by κ opioid receptors (< 2-fold decrease in affinity). However, this compound shows a 3-fold increase in affinity for μ opioid receptors, and therefore this compound exhibits about 4-fold lower selectivity compared to arodyn. Like arodyn, both compounds **11** and **12** show minimal AC inhibition at 10 μM. These results suggest that the agonist activity

at κ opioid receptors of compound **4** is most likely due to altered interactions with the receptors. One possibility is that the change in residue 3 (from Phe to Cha) may shift the position of Tic^1 in such a way that the acetyl group can form a hydrogen bond with the receptor, resulting in receptor activation.

Table 5.3: Pharmacological activities of [Tic^1,Cha^3]arodyn (**4**) and its analogs

Arodyn (**1**): Ac-Phe-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2

Peptides (modifications to arodyn)	K_i ± SEM (nM)[a]		K_i ratio (κ/μ)	AC % inhibition @ 10 μM[a,b]
	κ	μ		
1, Arodyn[c]	10.0 ± 3.0	1750 ± 130	1/175	12 ± 8
4 [Tic^1,Cha^3][d]	2.86 ± 0.81 (4)	240 ± 15 (4)	1/85	83 ± 4
11 [Tic^1][d]	7.95 ± 1.26 (4)	970 ± 70 (4)	1/122	10 ± 10 (2)
12 [Cha^3]	14.2 ± 3.9 (4)	660 ± 82	1/46	15 ± 10
13 [D-Tic^1,Cha^3]	9.55 ± 2.81 (4)	1250 ± 370	1/130	-10 ± 10 (4)
14 [Ppa^1,Cha^3]	12.1 ± 1.3 (4)	850 ± 190	1/70	-10 ± 11 (4)
15 [(S/R)-2-$MePpa^1$,Cha^3]	2.67 ± 0.79	1990 ± 250	1/745	5 ± 18 (4)
16 [(S/R)-Tna^1,Cha^3]	6.50 ± 0.82 (4)	1930 ± 700 (5)	1/297	-23 ± 6 (2)
17 [Ppa^1]	23.3 ±5.2 (4)	2780 ± 410	1/119	5 ± 12
18 [(S/R)-2-$MePpa^1$]	8.20 ± 0.99	4440 ± 300	1/541	11 ± 27
19 [(S/R)-Tna^1]	11.9 ± 2.9	3200 ± 480	1/269	-39 ± 8 (4)

[a]The results are expressed as the mean ± SEM % of 3 independent experiments except where noted; [b]Compared to Dyn A (1-13)NH_2 (100%); [c]From reference 11; [d]From reference 13.

D-Tic substitution in position 1 in compound **4** results in a compound (**13**) with 3.4- and 5-fold decreases in affinity for κ and μ opioid receptors, respectively, compared to **4** (Table 5.3). Unlike **4**, compound **13** does not exhibit efficacy. One possibility is that the change in configuration of the first amino acid alters a possible hydrogen bond of the N-terminal acetyl group with κ opioid receptors. Changing the stereochemistry of Phe^1 in

arodyn results in a similar decrease in affinity for κ opioid receptors; however, neither arodyn nor its D-Phe[1] analog exhibit significant efficacy in the AC assays.[10]

The Ac-Tic group in **4** was then substituted with Ppa, 2-MePpa, and Tna (compounds **14-16**, Table 5.3) to evaluate the effects of elimination of a possible hydrogen bond on opioid receptor affinity and selectivity as well as efficacy. Substitution of Ac-Tic in compound **4** with Ppa results in compound **14** with about 4-fold lower affinity for κ opioid receptors compared to **4**. The affinity for μ opioid receptors decreases by about 3.5-fold, and therefore this compound has almost identical selectivity for κ over μ opioid receptors as **4**. 2-MePpa and Tna were first incorporated into peptides as racemates and the affinity and efficacy initially determined for the resulting diastereomer peptide mixtures. The mixture of [(R/S)-2-MePpa[1],Cha[3]]arodyn (**15**) exhibits significantly higher κ receptor affinity than **14**, likely due to the effect of the methyl group on the orientation of the side chain. The effect of an α-methyl group on the orientation of the side chains has been observed in another potent κ opioid antagonist dynantin ([(2S)-Mdp[1]]Dyn A-(1-11)NH_2, Mdp = 2-methyl-3-(2',6'-dimethyl-4-hydroxyphenyl)propionic acid),[18] which has 4-fold higher affinity compared to its des-methyl analog. The individual isomers will need to be evaluated separately to determine how the stereochemistry affects κ receptor affinity. The affinity of the diastereomeric mixture for μ opioid receptors is similar to arodyn, and therefore, the mixture exhibits very high selectivity (745-fold) for κ over μ opioid receptors.

The κ receptor affinity of the mixture of [(S/R)-Tna[1],Cha[3]]arodyn (compound **16**) is intermediate between **14** and **15**. The μ opioid receptor affinity of **16** is similar to **15**, so that the selectivity of the mixture is higher than **14**, but lower than **15**. Unlike **4**, none

of these mixtures (**14-16**) shows any agonist activity in the functional assay. [(S/R)-Tna[1],Cha[3]]arodyn (**16**) actually appears to show some inverse agonist activity with stimulation of AC activity at 10 μM. The evaluation of the efficacy of the individual isomers of **15** is underway. The individual isomers of **16** will also be prepared and evaluated if the enantiomers of Tna can be resolved.

The corresponding Phe[3] analogs (**17-19**) of these three analogs **14-16** have lower affinities (2- to 3-fold) for κ opioid receptors compared to their corresponding Cha[3] analogs. Their affinities for μ opioid receptors also decrease, resulting in similar selectivity for κ over μ opioid receptors to their corresponding Cha[3] analogs. The mixture [(S/R)-2-MePpa[1]]arodyn (**18**) shows very high selectivity (541-fold) for κ over μ opioid receptors, similar to the Cha[3] analog **15**. As expected, these compounds exhibit no significant inhibition of AC activity. The mixture **19** appears to show inverse agonist activity, similar to the corresponding Cha[3] analog **16**. This mixture was then examined for dose-dependent stimulation of AC activity under basal conditions (without added forskolin). Under these conditions, the stimulation of the AC activity is enhanced over basal AC activity. This mixture exhibits dose-dependent inverse agonist activity (EC_{50} = 100 nM) in the initial assays. The efficacy of the individual isomers of **18** will be evaluated shortly.

5.3 Conclusions

In conclusion, while arodyn is a potent and selective neutral κ opioid receptor antagonist, analogs exhibiting both inverse agonist and agonist activity at κ opioid receptors have been identified.

Kappa opioid receptors generally tolerate bulky aromatic amino acids at position 3 of arodyn analogs with minimal loss in affinity. Substitution of Nal(2') in both positions 1 and 3 (compound **2**) results in a 2-fold increase in κ opioid receptor affinity. However, incorporation of a bulky aromatic amino acid at position 1 and/or 3 generally decreases selectivity for κ over μ opioid receptors compared with arodyn by decreasing affinity for κ receptors and/or increasing affinity for μ opioid receptors. Compound **9** ([Nal(2')^{1}]arodyn) shows only 17-fold selectivity for κ over μ opioid receptors. A bulky aromatic ring in position 3 (such as Trp and Nal(1'/2')) in the arodyn analogs appears to be important for inverse agonist activity, while the residue in position 1 is not important for inverse agonist activity. [Nal(2')^{1,3}]arodyn (**2**) and [Nal(2')^{3}]arodyn (**6**) show dose-dependent inverse agonist activity with nanomolar potency (EC_{50} = 84 and 9.0 nM, respectively) at κ opioid receptors.

[Tic^{1},Cha^{3}]arodyn (**4**) and its Lys^{4} analog (**5**) are the only two arodyn analogs that show almost full agonist activity at κ opioid receptors.[13] The combined modifications of both the first and third residues of arodyn not only increase κ opioid receptor affinity but also efficacy. The enhanced efficacy may be due to the changes in residue 3 (from Phe to Cha) which could shift the position of Tic^{1} in the binding site such that the hydrogen bonding of the N-terminal acetyl group with a group on the receptor could change the receptor conformation and result in receptor activation. Changing the configuration of the first amino acid Tic could alter or eliminate a possible hydrogen bond between the N-terminal acetyl group with κ opioid receptors and [D-Tic^{1},Cha^{3}]arodyn (**13**) is no longer an agonist.

Substitution of the N-terminal acetyl moiety (Ac-Tic[1]) in [Tic[1],Cha[3]]arodyn with Ppa, 2-MePpa, and Tna (compounds **14-16**) can eliminate the H-bonding capability, and therefore eliminate the agonist activity. Therefore, these compounds do not exhibit agonist activity. These analogs generally exhibit higher affinity for κ opioid receptors and similar affinity for μ opioid receptors compared to arodyn. The mixture of [(S/R)-2-MePpa[1],Cha[3]]arodyn (**15**) exhibits the highest affinity (K_i = 2.7 nM) and selectivity (K_i ratio (κ/μ) = 1/745) for κ opioid receptors. The Phe[3] analogs (**17-19**) of these three analogs **14-16** have lower affinity (2- to 3-fold) but similar selectivity for κ opioid receptors compared to their corresponding Cha[3] analogs. The two enantiomers of 2-MePpa have been resolved and incorporated into the peptides as single isomers (compounds **20-23**, Table 5.4). The pharmacological activities of these peptides will be determined shortly.

Table 5.4: Analytical data for compounds **20-23**

Peptides (Modifications to arodyn)	HPLC (t_R (min)/% Purity)		ESI-MS (*m/z*)	
	System 1[a]	System 2[b]	Calculated	Observed
20 [(S)-2-MePpa[1],Cha[3]]	29.73/100	30.06/100	$[M+4H]^{4+}$=375.2 $[M+3H]^{3+}$=500.0	$[M+4H]^{4+}$=375.2 $[M+3H]^{3+}$=500.0
21 [(R)-2-MePpa[1],Cha[3]]	29.79/100	29.66/100	$[M+4H]^{4+}$=375.2 $[M+3H]^{3+}$=500.0	$[M+4H]^{4+}$=375.2 $[M+3H]^{3+}$=500.0
22 [(S)-2-MePpa[1]]	27.79/100	27.73/100	$[M+4H]^{4+}$=373.7 $[M+3H]^{3+}$=498.0	$[M+4H]^{4+}$=373.7 $[M+3H]^{3+}$=498.0
23 [(R)-2-MePpa[1]]	26.54/100	27.09/100	$[M+4H]^{4+}$=373.7 $[M+3H]^{3+}$=498.0	$[M+4H]^{4+}$=373.7 $[M+3H]^{3+}$=498.0

[a]System 1: Solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in acetonitrile, 5-50% solvent B over 45 min. [b]System 2: Solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in methanol, 5-86% solvent B over 45 min.

5.4 Experimental section

5.4.1 Materials

Fmoc-Nal(2')-OH was purchased from Peptides International (Louisville, KY). Fmoc-Nal(1')-OH was purchased from Synthetech (Albany, OR). Ppa and racemic 2-MePpa and Tna were purchased from Sigma-Aldrich (Milwaukee, WI). The sources of other materials are listed in Chapter 3.

5.4.2 Synthesis, purification and analysis of peptides

All of the peptides were synthesized on the Fmoc-PAL-PEG-PS resin by standard solid-phase peptide synthesis (SPPS). The desired Fmoc-protected amino acids or acid were coupled to the growing peptide chain with PyBOP, HOBt, and DIEA (4/4/10) in DMF for 2 h. The peptides were then cleaved from the resin using Reagent B (85% TFA, 5% phenol, 5% water, and 2% triisopropylsilane) at room temperature for 2 h.[14] The crude peptides were purified by preparative reversed-phase HPLC using a linear gradient of 15-50% MeCN containing 0.1% TFA over 35 min, at a flow rate of 20 mL/min. The purification was monitored by UV absorbance at 214 nm. The purity of the final peptides was verified using analytical HPLC in two different solvent systems (see Tables 5.1 and 5.4). Molecular weights of the compounds were determined by electrospray ionization mass spectrometry (ESI-MS, Tables 5.1 and 5.4). For the detailed procedures for peptide synthesis, purification and analysis, please refer to Chapters 3 and 4.

5.4.3 Resolution of 2-MePpa

2-MePpa-Phe-NH_2 was synthesized on the Fmoc-PAL-PEG-PS resin (0.20 mmol/g, 3 g) by standard procedures using PyBOP, HOBt, and DIEA as coupling reagents followed by pure TFA cleavage. The TFA was then removed by air, and the two diastereomers of 2-MePpa-Phe-NH_2 were purified to > 98% de using reversed-phase HPLC (30-50% MeOH over 200 min at 20 mL/min). The solvent was then removed by lyophilization to afford 76 mg (S)-2-MePpa-Phe-NH_2 (84% yield, t_R = 16.31 min, 100% purity, 15-40% acetonitrile containing 0.1% TFA over 25 min; $[M+Na]^+$ = 333.15, $[2M+Na]^+$ = 643.31) and 58 mg (R)-2-MePpa-Phe-NH_2 (62% yield, t_R = 17.00 min, 99.4% purity, 15-40% acetonitrile containing 0.1% TFA over 25 min; $[M+Na]^+$ = 333.15, $[2M+Na]^+$ = 643.31), respectively.

The isolated diastereomer (S)-2-MePpa-Phe-NH_2 (the isomer with the shorter retention time in reversed phase HPLC, 76 mg) and 3 M HCl (60 mL) were heated under reflux for 8 h. The completeness of the hydrolysis reaction was monitored by HPLC (5-50% acetonitrile containing 0.1% TFA over 45 min). After the mixture cooled to room temperature, the acid was extracted with 50 mL ethyl ether 3 times. The combined ethyl ether layers were back-extracted with 3 M HCl (30 mL X 2), and the ether solution was washed with brine, dried over magnesium sulfate and evaporated to produce (S)-2-MePpa (23.8 mg, yield 59%, $[\alpha]_D$ +19.89^o (c 0.0603, $CHCl_3$) [Lit.[19] $[\alpha]_D$ +23.51^o]. The other enantiomer ((R)-2-MePpa) was obtained in the same manner (26.8 mg, yield 87%; $[\alpha]_D$ – 18.42^o (c 0.1407, $CHCl_3$) [Lit.[19] $[\alpha]_D$ –24.56^o]).

5.4.3 Pharmacological assays

The radioligand binding assays and cyclase activity screening assays have been described in Chapter 4. The peptide concentration was varied from 0.1-10,000 nM in 10-fold dilutions to determine the potencies of the peptides in the AC assay.

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Chapter 6. Design, Synthesis and Biological Activity of Arodyn Analogs Cyclized by Lactam Bond Formation

*Note that the compound numbers used in this chapter are only applicable within this specific chapter.

6.1 Introduction

In the last chapter, we described the structure-activity relationships (SAR) of linear aroydn analogs exhibiting agonist and inverse agonist activity for kappa (κ) opioid receptors. This chapter focuses on the design and synthesis of cyclic arodyn analogs by side chain lactam bond formation, and the study of the effects of conformational constraint of arodyn analogs on affinity, selectivity, and efficacy for κ opioid receptors.

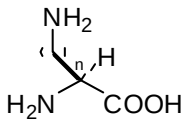
The linear peptide arodyn can adopt numerous conformations, and the biologically active conformation(s) of this peptide are not known. Compared with linear peptides, cyclic peptides generally show reduced conformational mobility, which is especially useful for the study of receptor-ligand interactions.[1-3] Constraining the conformation could result in higher potency and/or selectivity,[3] as a pre-organized conformation can decrease the entropy penalty for receptor binding. Cyclic peptides are also often more stable to peptidases than linear peptides.[4-6] Arodyn itself shows a very short half-life of less than 2 minutes in rat brain homogenate.[6] Furthermore, cyclization may increase membrane transport as shown for some cyclic Dyn A analogs.[6] Hence cyclic peptides can represent promising lead compounds for future development.

Although a variety of conformationally constrained peptide agonists for κ-opioid receptors have been identified,[2, 7] the search for conformationally constrained peptide antagonists for κ receptors has been quite limited. To date there are only two cyclic Dyn A analogs reported with antagonist activity at κ opioid receptors.[8, 9] Based on the novel acetylated chimeric Dyn A analog venorphin,[10] our laboratory designed and synthesized cyclodyn (*cyclo*^{N,5}[Trp^3,Trp^4,Glu^5]Dyn A-(1-11)NH_2), the first cyclic Dyn A analog with antagonist activity at κ opioid receptors.[8] Recently, a Dyn A analog cyclized in the

"address"[11] domain, [N^{α}-benzyl-Tyr^{1},*cyclo*(D-Asp^{5},Dap^{8})]Dyn A-(1-11)NH_2, was also identified in our laboratory as a novel κ opioid receptor antagonist.[9]

Arodyn is a potent and highly selective antagonist for κ opioid receptors,[12] and therefore it provides a very good lead structure for the synthesis of conformationally constrained analogs with antagonist activity. We first synthesized cyclic arodyn analogs through cyclization between an amine and carboxylic acid by lactam bond formation. Cyclization was performed between the side chain of D/L-Asp and the side chain of Dap (2,3-diaminopropionic acid), Dab (2,4-diaminobutyric acid), Orn, or Lys. The structures of Dap, Dab, and Orn are shown in Figure 6.1.

Figure 6.1: Structures of Dap, Dab, and Orn



Dap, n=1
Dab, n=2
Orn, n=3

The residues for cyclization were chosen based on the SAR of linear arodyn analogs. Previously an alanine scan showed that positions 2, 5, and 8 are not crucial for the binding of arodyn to κ opioid receptors.[13] By cyclizing between noncritical residues, the conformational mobility of important residues can be restricted. Based on this rationale and the sequence of arodyn, we chose to introduce cyclizations between residues 2 and 5 (compounds **2**-**4**), 2 and 8 (compounds **5** and **6**), and 5 and 8 (compounds **7** and **8**, Table 6.1). We used different configurations of amino acids and/or positions for cyclizations because different cyclizations can induce different

conformations, and therefore could result in ligands with different affinities and/or selectivities for κ opioid receptors. In compounds **2-4**, Ile, which is found in Dyn A, was incorporated in position 8 rather than D-Ala to maintain the same stereochemistry of this residue in all of the cyclic analogs.

Table 6.1: Structures of cyclic arodyn analogs and the corresponding linear peptides

#	Compound (Modifications to arodyn)	Structure
1	Arodyn	Ac-Phe-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Pro-Lys-NH_2
Cyclic arodyn analogs		
2	*cyclo*[D-Asp^2,Dap^5,Ile^8]	Ac-Phe-D-Asp-Phe-Arg-Dap-Arg-Arg-Ile-Arg-Pro-Lys-NH_2
3	*cyclo*[Asp^2,Dap^5,Ile^8]	Ac-Phe-Asp-Phe-Arg-Dap-Arg-Arg-Ile-Arg-Pro-Lys-NH_2
4	*cyclo*[Asp^2,Lys^5,Ile^8]	Ac-Phe-Asp-Phe-Arg-Lys-Arg-Arg-Ile-Arg-Pro-Lys-NH_2
5	*cyclo*[D-Asp^2,Dap^8]	Ac-Phe-D-Asp-Phe-Arg-Leu-Arg-Arg-Dap-Arg-Pro-Lys-NH_2
6	*cyclo*[Asp^2,Dap^8]	Ac-Phe-Asp-Phe-Arg-Leu-Arg-Arg-Dap-Arg-Pro-Lys-NH_2
7	*cyclo*[D-Asp^5,Dap^8]	Ac-Phe-Phe-Phe-Arg-D-Asp-Arg-Arg-Dap-Arg-Pro-Lys-NH_2
8	*cyclo*[Asp^5,Dap^8]	Ac-Phe-Phe-Phe-Arg-Asp-Arg-Arg-Dap-Arg-Pro-Lys-NH_2
Linear analogs		
9	[D-Asn^2,Dap(Ac)^5,Ile^8]	Ac-Phe-D-Asn-Phe-Arg-Dap(Ac)-Arg-Arg-Ile-Pro-Lys-NH_2
10	[Asn^2,Dap(Ac)^5,Ile^8]	Ac-Phe-Asn-Phe-Arg-Dap(Ac)-Arg-Arg-Ile-Pro-Lys-NH_2
11	[D-Asn^2,Dap(Ac)^8]	Ac-Phe-D-Asn-Phe-Phe-Arg-Leu-Arg-Arg-Dap(Ac)-Pro-Lys-NH_2
12	[Asn^2,Dap(Ac)^8]	Ac-Phe-Asn-Phe-Phe-Arg-Leu-Arg-Arg-Dap(Ac)-Pro-Lys-NH_2
13	[D-Asn^5,Dap(Ac)^8]	Ac-Phe-Phe-Phe-Arg-D-Asn-Arg-Arg-Dap(Ac)-Pro-Lys-NH_2
14	[Asn^5,Dap(Ac)^8]	Ac-Phe-Phe-Phe-Arg-Asn-Arg-Arg-Dap(Ac)-Pro-Lys-NH_2

Linear analogs corresponding to these cyclic peptides (compounds **9-14**, Table 6.1) were also synthesized to determine whether any changes in pharmacological activity were due primarily to the conformational constraint. In these analogs, the D/L-Asp was

replaced by D/L-Asn, while the Dap was acetylated (Dap(Ac)) to avoid the introduction of any charged residues.

6.2 Results and discussion

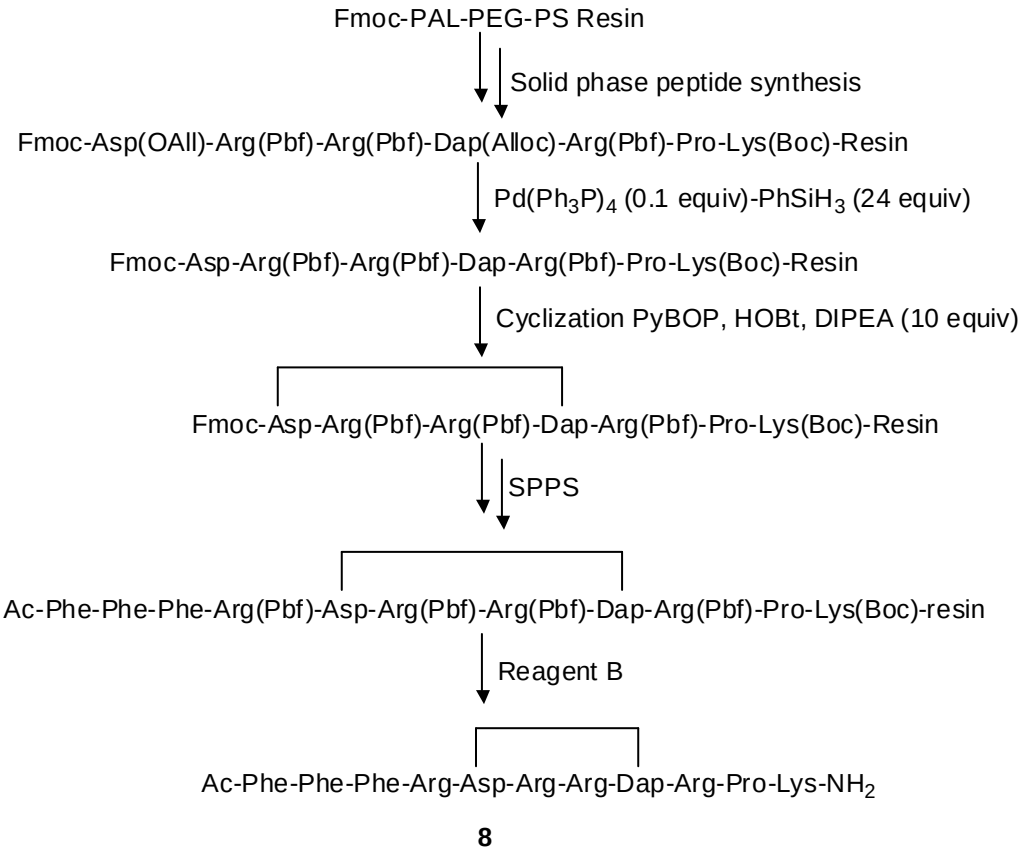
6.2.1 Chemistry

The synthesis of side chain-to-side chain cyclic peptides requires selective removal of protecting groups from the side chains of the amino acids involved in the cyclization without affecting the other side chain protecting groups. We used the method developed previously in our laboratory[14] which involves allyl-type protecting groups, namely the allyl ester and the Alloc (allyloxycarbonyl) group, for D/L-Asp and Dap/Dab/Orn/Lys, respectively. These two protecting groups can be removed with tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) and a nucleophile, thereby providing a three-dimensional orthogonal protection strategy.

The cyclizations were carried out after the Fmoc-Asp(OAll)-OH (Fmoc = 9-fluorenylmethoxycarbonyl) was coupled, but before Fmoc deprotection with piperidine (Scheme 6.1). This way base-catalyzed aspartimide formation involving the allyl ester on the Asp side chain could be minimized.[14] Cyclization reactions are conformationally dependent and are usually relatively slow. Therefore the reactions were allowed to run for 2 days with larger equivalents (10/10/20) of the activating reagents benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBt), and *N*,*N*-diisopropylethylamine (DIEA) than were used for regular amino acid coupling (4/4/8). The cyclization reactions appeared to be complete for these cyclic peptides as determined by the ninhydrin test.[15] Any trace amount of free amine was

acetylated with a large excess of acetic anhydride (20-fold) in DMF for 30 min. The Fmoc group was then removed and the peptide synthesis was continued until the entire peptide sequence was assembled on the resin.

Scheme 6.1: Synthesis of cyclic peptide **8**



8

The cyclic peptides and their linear analogs were purified to >98% purity as determined by high-performance liquid chromatography (HPLC). The molecular weights of these peptides were determined by electrospray ionization mass spectrometry (ESI-MS). The analytical data for these peptides are shown in Table 6.2.

Table 6.2: Analytical data for the cyclic arodyn analogs and the corresponding linear peptides

Peptides	HPLC (t_R (min)/% Purity)		ESI-MS (*m/z*)	
	System 1[a]	System 2[b]	Calculated	Observed
Cyclic analogs				
3	16.63/100	30.05/100	$[M+3H]^{3+}$=500.6 $[M+2H]^{2+}$=750.4 $[M+4H]^{4+}$=376.0	$[M+3H]^{3+}$=500.6 $[M+2H]^{2+}$=750.5 $[M+4H]^{4+}$=376.0
4	17.33/100	32.24/100	$[M+3H]^{3+}$=514.6 $[M+4H]^{4+}$=386.2 $[M+2H]^{2+}$=771.5	$[M+3H]^{3+}$=514.6 $[M+4H]^{4+}$=386.2 $[M+2H]^{2+}$=771.5
5	14.65/100	29.71/100	$[M+4H]^{4+}$=375.7 $[M+3H]^{3+}$=500.6	$[M+4H]^{4+}$=375.7 $[M+3H]^{3+}$=500.6
6	16.18/100	31.90/100	$[M+4H]^{4+}$=375.7 $[M+3H]^{3+}$=500.6	$[M+4H]^{4+}$=375.7 $[M+3H]^{3+}$=500.6
7	19.90/100	33.67/100	$[M+3H]^{3+}$=512.0 $[M+4H]^{4+}$=384.2 $[M+2H]^{2+}$=767.4	$[M+3H]^{3+}$=512.0 $[M+4H]^{4+}$=384.2 $[M+2H]^{2+}$=767.5
8	20.29/100	33.60/100	$[M+3H]^{3+}$=512.0 $[M+2H]^{2+}$=767.4 $[M+4H]^{4+}$=384.5	$[M+3H]^{3+}$=511.9 $[M+2H]^{2+}$=767.4 $[M+4H]^{4+}$=384.4
Linear analogs				
9	14.22/100	26.32/100	$[M+4H]^{4+}$=390.5 $[M+3H]^{3+}$=520.3 $[M+2H]^{2+}$=780.0	$[M+4H]^{4+}$=390.5 $[M+3H]^{3+}$=520.3 $[M+2H]^{2+}$=780.0
10	16.69/100	30.20/100	$[M+4H]^{4+}$=390.5 $[M+3H]^{3+}$=520.3	$[M+4H]^{4+}$=390.5 $[M+3H]^{3+}$=520.3
11	17.37/100	33.63/100	$[M+4H]^{4+}$=390.5 $[M+3H]^{3+}$=520.3	$[M+4H]^{4+}$=390.5 $[M+3H]^{3+}$=520.3
12	18.73/100	37.34/100	$[M+4H]^{4+}$=390.5 $[M+3H]^{3+}$=520.3	$[M+4H]^{4+}$=390.5 $[M+3H]^{3+}$=520.3
13	18.82/100	32.09/100	$[M+4H]^{4+}$=399.2 $[M+3H]^{3+}$=530.0	$[M+4H]^{4+}$=399.2 $[M+3H]^{3+}$=532.0
14	20.04/100	34.05/100	$[M+4H]^{4+}$=399.2 $[M+3H]^{3+}$=530.0	$[M+4H]^{4+}$=399.2 $[M+3H]^{3+}$=532.0

[a]System 1: 5-50% Solvent B over 45 min (Solvent A= H_2O; Solvent B= MeCN, both containing 0.1% TFA). [b]System 2: 5-50% Solvent B over 45 min (Solvent A= H_2O; Solvent B= MeOH, both containing 0.1% TFA).

6.2.2 Pharmacology

The peptides were examined for their κ opioid receptor affinity as described previously using Chinese hamster ovary (CHO) cells stably expressing cloned opioid receptors (Table 6.3).[16] Most of the cyclic peptides evaluated showed much lower affinity

for κ opioid receptors compared to arodyn. The cyclizations in the N-terminal "message" sequence of arodyn involving residues 2 and 5 were unfavorable for κ opioid receptor binding. *cyclo*[Asp^2,Dap^5,Ile^8]arodyn (**3**) shows 58-fold lower affinity compared to arodyn, and *cyclo*[Asp^2,Lys^5,Ile^8]arodyn (**4**), with substitution of Lys at position 5, exhibits similar low affinity. The linear analog [Asn^2,$Dap(Ac)^5$,Ile^8]arodyn (**10**) also exhibits much lower affinity (23-fold) for κ opioid receptors compared with arodyn. This suggests that the loss of κ receptor affinity for compounds **3** and **4** is largely due to the introduction of the corresponding functionalities used for cyclization. *cyclo*[D-Asp^2,Dap^5,Ile^8]arodyn (**2**) shows very low affinity (K_i = 3000 nM) for κ opioid receptors. Its linear analog, [D-Asn^2,$Dap(Ac)^5$,Ile^8]arodyn (**9**), exhibits much higher affinity (K_i = 60 nM) than the cyclic analog (Table 6.3), suggesting that the decreased affinity of **2** is mainly due to the constrained conformation induced by cyclization. It shows that in the linear analogs subsitution of D-Asn is better tolerated than Asn in position 2 of arodyn for κ opioid receptors.

Longer-range cyclizations involving residues 2 and 8 of arodyn resulted in similar large decreases in κ opioid receptor affinity. *cyclo*[D-Asn^2,Dap^8]arodyn (**5**) and *cyclo*[Asn^2,Dap^8]arodyn (**6**) show 68- and 37-fold lower affinity respectively for κ opioid receptors compared to arodyn, suggesting that the conformations induced by these types of cyclizations are not favorable for κ receptor binding. The evaluation of mu (μ) affinity of these cyclic peptides is underway.

Table 6.3: Preliminary Pharmacological activity of cyclic arodyn analogs and the corresponding linear peptides

Compound	K_i (nM)[a]		K_i ratio (κ/μ)	AC % inhibition @ 10 μM[a,b]
	κ	μ		
1, arodyn[c]	10.0 ± 3.0	1750 ± 130	1/175	12 ± 8
Cyclic analogs				
2	3000 ± 270	ND[d]	ND	
3	580 ± 150	ND	ND	-4 ± 7
4	858 ± 283 (4)	ND	ND	-5 ± 5
5	677 ± 38	ND	ND	-5 ± 9
6	373 ± 43	ND	ND	16 ± 6
7	55.3 ± 15.1	>10,000	1/>182	-20 ± 10
8	19.7 ± 1.5 (2)	>10,000	1/>508	-11 ± 7
Linear analogs				
9	60.3 ± 2.8	ND	ND	4 ± 22
10	227 ± 13	ND	ND	-23 ± 11

[a]The results are expressed as the mean ± SEM of 3 independent experiments except where noted. [b]Compared to Dyn A(1-13)NH_2 taken as 100% inhibition. [c]From reference 12. [d]Not determined.

Cyclizations in the C-terminal "address" sequence of arodyn involving residues 5 and 8 are favorable for κ opioid receptor binding (Table 6.3). In preliminary assays *cyclo*[Asp^5,Dap^8]arodyn (**8**) shows only 2-fold lower affinity (K_i = 20 nM) compared to arodyn (**1**, K_i = 10 nM). This is the first cyclic arodyn analog that exhibits high affinity at κ opioid receptors and is an excellent lead peptide. This compound shows negligible binding affinity for μ opioid receptors (K_i > 10 μM), and therefore the selectivity for κ over μ opioid receptors of this cyclic peptide appears to be higher than arodyn. *cyclo*[D-Asp^5,Dap^8]arodyn (**7**), with a D-amino acid at position 5, shows about 2.5-fold lower affinity for κ opioid receptors compared to **8**, indicating a stereochemical preference for this residue in the cyclic constraint. Like **8**, this peptide showed negligible affinity for μ opioid receptors.

These arodyn analogs were evaluated for their intrinsic activity at 10 μM in the adenylyl cyclase (AC) inhibition assay using cloned rat κ opioid receptors stably expressed on CHO cells (Table 6.3).[17] Similar to arodyn, these analogs exhibit negligible or very low efficacy. Further evaluation of peptides **7** and **8** for antagonist activity will be conducted shortly to determine their ability to reverse the agonist activity of Dyn A-(1-13)NH_2.

6.3 Conclusions and future studies

In conclusion, we designed and synthesized a series of cyclic arodyn analogs. Cyclizations involving lactam bond formation between amino acid side chains were introduced in three different regions: in the N-terminal "message" sequence, the C-terminal "address" domain, and via longer-range cyclization. Several analogs show substantially lower affinity at κ opioid receptors. In contrast, preliminary results show that *cyclo*[Asp^5,Dap^8]arodyn (**8**) retains similar affinity (K_i = 20 nM) for κ opioid receptors and exhibits much lower affinity for μ opioid receptors (K_i > 10 μM) than arodyn. This is the first cyclic arodyn analog that exhibits significant affinity and very high selectivity for κ opioid receptors. *cyclo*[D-Asp^5,Dap^8]arodyn (**7**), with a D-amino acid at position 5, shows about 2.5-fold lower affinity compared to **8**; this compound also exhibits very low affinity for μ opioid receptors (K_i > 10 μM). Neither of these two analogs exhibits any inhibition of AC activity, similar to arodyn. Thus we have successfully identified the first generation of side chain-to-side chain cyclic arodyn analogs as potential antagonists for κ opioid receptors. *cyclo*[Asp^5,Dap^8]arodyn is an interesting lead peptide for future modifications.

Since this lead compound **8** has not been optimized, the first step towards a systematic SAR study was to modify the ring size. Such a modification may change the backbone conformation and orientation of the side chains and will provide information with regard to the optimal ring size required for receptor interaction. The easiest way to vary the ring size is to change the amino acid Dap[8] to Dab and Orn. These two compounds (**15** and **16**) have been synthesized and purified (Table 6.4) and are currently undergoing pharmacological evaluation. Other modifications can be introduced based on the SAR analysis of linear arodyn analogs described in previous chapters to further improve the pharmacological activities of these analogs.

Table 6.4: Analytical data for the cyclic arodyn analogs **15** and **16**

Peptides	HPLC (t_R (min)/% Purity)		ESI-MS (*m/z*)	
	System 1[a]	System 2[b]	Calculated	Observed
15	19.37/100.0	23.23/100.0	$[M+4H]^{4+}$=387.7 $[M+3H]^{3+}$=516.6	$[M+4H]^{4+}$=387.7 $[M+3H]^{3+}$=516.6
16	19.32/97.9	23.23/97.6	$[M+4H]^{4+}$=391.2 $[M+3H]^{3+}$=521.3	$[M+4H]^{4+}$=391.2 $[M+3H]^{3+}$=521.3

[a]System 1: 5-50% Solvent B over 45 min (Solvent A= H_2O; Solvent B= MeCN containing 0.1% TFA). [b]System 2: 5-50% Solvent B over 45 min (Solvent A= H_2O; Solvent B= MeOH containing 0.1% TFA).

6.4 Experimental section

6.4.1 Materials

Fmoc-D-Asp(OAll)-OH was purchased from Senn Chemical (San Diego, CA). Fmoc-Asp(OAll)-OH, Fmoc-Dap(Alloc), Fmoc-Dab(Alloc)-OH, and Fmoc-Orn(Alloc)-OH were purchased from Peptides International (Louisville, KY). Fmoc-Lys(Alloc)-OH was purchased from Applied Biosystems (Foster City, CA). $Pd(PPh_3)_4$ and phenylsilane ($PhSiH_3$) were purchased from Acros Chemical Co. (Pittsburg, PA). Tetrahydrofuran

(THF) was purchased from Fisher Scientific (Hampton, NH). Sodium diethyldithiocarbamate trihydrate was purchased from Sigma-Aldrich (Milwaukee, WI). The sources of other materials are listed in Chapter 3.

6.4.2 Synthesis

Synthesis of cyclic peptides. The cyclic peptides were synthesized by solid phase peptide synthesis (SPPS) following the procedure developed in our laboratory.[14] All of the peptides were synthesized on the Fmoc-PAL-PEG-PS resin (PAL-PEG-PS: peptide amide linker-poly(ethylene glycol)-polystyrene, 0.20 mmol/g, 500 mg). The synthesis of the peptide **7** (*cyclo*[Asp^5,Dap^8]arodyn) is shown in Scheme 6.1 as an example. The side chains of Asp^5 and Dap^8 that were involved in the cyclization were protected as the allyl ester and Alloc group, respectively. The side chain of Lys in position 11 was protected by Boc (*tert*-butyloxycarbonyl) and Arg by Pbf (2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl). The protected peptide Fmoc-Asp(OAll)-Arg(Pbf)-Arg(Pbf)-Dap(Alloc)-Arg(Pbf)-Pro-Lys(Boc)-resin was first assembled according to the procedures described in Chapter 3, except that a 2-fold excess of Fmoc-Asp(OAll)-OH and Fmoc-Dap(Alloc)-OH were used for coupling (2 hour reaction). The OAll and Alloc groups were selectively deprotected by $Pd(PPh_3)_4$ (23 mg, 0.02 mmol, 0.1 equiv) and $PhSiH_3$ (430 mg, 4.8 mmol, 24 equiv) in dichloromethane (DCM, 2 X 30 min).[18] The reaction was carried out under argon atmosphere to avoid oxidation of the catalyst. After the reaction, the peptide-resin was washed with DCM (5 X 2 min), THF (4 X 2 min), DCM (3 X 2 min), *N*,*N*-dimethylformamide (DMF) (3 X 2 min), 0.5% DIEA in DMF (3 X 2 min), 0.02 M sodium diethyldithiocarbamate in DMF (3 X 15 min), and DMF (5 X 2 min).[19, 20] The

free carboxylic acid group of Asp and the amino group of Dap were then cyclized using a 10-fold excess of PyBOP and HOBt, and 20-fold excess DIEA (0.2 M in DMF). The reaction was allowed to run for 2 d and the completion of cyclization was monitored by the ninhydrin test.[15] Any trace amount of free amine was acetylated with a large excess of acetic anhydride (20-fold) in DMF for 30 min. The Fmoc group was then removed and the peptide synthesis was continued until the entire peptide sequence was assembled on the resin.

Synthesis of linear analogs. The linear peptides were assembled on the resin using Fmoc-Asn(Trt)-OH (Trt = trityl) and Fmoc-D-Asn(Trt)-OH in place of Fmoc-Asp(OAll)-OH and Fmoc-D-Asp(OAll)-OH, respectively. The Alloc group on Dap was selectively deprotected by $Pd(PPh_3)_4$ and phenylsilane in DCM as described above. The free amine was then acetylated using acetic anhydride (20-fold) in DMF for 30 min.

6.4.3 Cleavage, purification, and analysis

The peptides were cleaved from the resin using Reagent B (85% TFA, 5% phenol, 5% water, and 2% triisopropylsilane) at room temperature for 2 h.[21] The crude peptides were purified by preparative reversed-phase HPLC using a linear gradient of 15-50% MeCN containing 0.1% TFA over 35 min at a flow rate of 20 mL/min. The purification was monitored by UV absorbance at 214 nm. The purity of the final peptides was verified using analytical HPLC in two different solvent systems (see Table 6.2). Molecular weights of the compounds were determined by ESI-MS. For the detailed procedures for peptide synthesis, purification and analysis, please refer to Chapters 3 and 4.

6.4.4 Pharmacological assays

Radioligand binding assays were performed using cloned rat κ and μ opioid receptors stably expressed in CHO cells as described in Chapter 4. The peptides were evaluated for their ability to inhibit the synthesis of cAMP by adenylyl cyclase as described in Chapter 4.

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Chapter 7. Design and Synthesis of Arodyn Analogs Cyclized by Ring-Closing Metathesis

*Note that the compound numbers used in this chapter are only applicable within this specific chapter.

7.1 Introduction

In the last chapter, we described the design, synthesis and pharmacological activity of arodyn analogs cyclized by lactam bond formation. This chapter is focused on the design and synthesis of arodyn analogs cyclized by ring-closing metathesis (RCM), and the study of the effects of the conformational constraint on the affinity, selectivity, and efficacy of the arodyn analogs for kappa (κ) opioid receptors.

As a potent and highly selective antagonist for κ opioid receptors, arodyn provides an excellent lead structure for the synthesis of conformationally constrained analogs with antagonist activity. Previously, we synthesized several cyclic arodyn analogs through lactam bond formation between amine and carboxylic acid side chains (see Chapter 6). The conformational constraints achieved for most of these cyclic peptides were not compatible with κ opioid receptor binding. One compound, *cyclo*[Asp^5,Dap^8]arodyn, retains similar affinity (K_i = 20 nM) as arodyn for κ opioid receptors and exhibits very higher selectivity (> 500-fold) for κ over μ opioid receptors. This was the first cyclic arodyn analog that exhibits significant κ receptor affinity and very high selectivity for κ opioid receptors.

Our current interest is in the design and synthesis of cyclic arodyn analogs utilizing RCM. RCM has emerged as a very useful method of making cyclic organic compounds as well as cyclic peptides.[1-4] Cyclization of peptides through RCM offers several advantages over the conventional methods of cyclization, i.e. through amide or disulfide bond formation. The resultant carbon-carbon bond is more stable than an amide or disulfide bond.[3, 5] Replacement of the disulfide bond in biologically active compounds can increase metabolic stability, as the double bond is stable to reducing conditions in

biological systems.[5] Furthermore, in contrast to cyclizations via amide or disulfide bond formation, side chain functionalities can be retained in cyclizations by RCM by appropriate choice of amino acids for cyclizations. In some cases the pharmacological activity of cyclic peptides is very different from linear analogs, mainly because of the change of functionality used for cyclization.[6] As shown in Chapter 6, *cyclo*[Asp^2,Dap^5,Ile^8]arodyn shows about 58-fold lower affinity compared to arodyn, partially due to such a change in functionalities for cyclization, since the linear analog, [Asn^2,Dap(Ac)^5,Ile^8]arodyn, also exhibits much lower affinity (23-fold) for κ opioid receptors compared to arodyn. In another example, a series of [N,5] cyclic Dyn A analogs and their corresponding linear analogs show considerable losses in affinity for opioid receptors compared to Dyn A,[6] suggesting that the functionalities used for cyclizations are not tolerated by κ opioid receptors. Since the RCM reaction only requires two alkene groups, we can easily modify the side chains to maintain functionalities similar to the parent peptide. For example, the side chain of Leu can be substituted with AllGly for cyclization without changing the aliphatic nature of the side chain. The side chain of Phe can be substituted with Tyr(All) for cyclization to maintain an aromatic functionality.

Cyclization of peptides by RCM has been applied to the synthesis of a few analogs of the opioid peptides dermorphin and the enkephalins.[7-9] Some of these cyclic analogs showed potent activity for both μ and δ opioid receptors. In contrast, there are no reports of longer opioid peptides such as Dyn A analogs cyclized through RCM. Here we utilize several different strategies for the synthesis of cyclic arodyn analogs by RCM.

7.2 Results and discussion

7.2.1 Ligand design

The analogs of arodyn cyclized by RCM were designed based on the structure-activity relationships (SAR) of linear analogs. The contributions of each residue of arodyn for κ opioid receptor affinity have been evaluated by an alanine scan (Figure 7.1).[10] In the N-terminal "message" sequence, Phe^1, Phe^3, and Arg^4 were found to contribute to the interactions between arodyn and κ opioid receptors.[10] Substitution of Phe^2 with Ala does not affect the κ opioid receptor affinity, but this substitution increases the selectivity for κ opioid receptors by decreasing μ opioid receptor affinity.[10] In the C-terminal "address" sequence, the basic residues (Arg^6, Arg^7, Arg^9, and Lys^{11}) contribute to κ opioid receptor affinity.[10] The des-acetyl analog exhibits 4.5-fold lower affinity for κ opioid receptors, indicating that a basic amine is detrimental for the κ opioid receptor binding of arodyn.[10] Substitution of Phe^1 with NMePhe in arodyn results in a 2-fold increase in κ opioid receptor affinity as well as a 6-fold increase in κ over μ opioid receptor selectivity compared to arodyn,[10] probably due to a change in the peptide backbone conformation.

Figure 7.1: SAR of arodyn.

Ac-Phe-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2

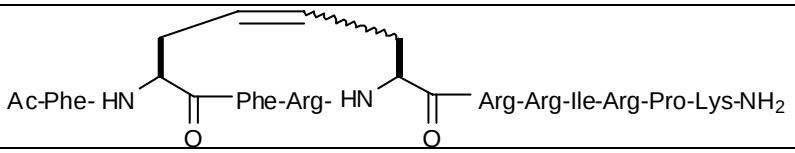
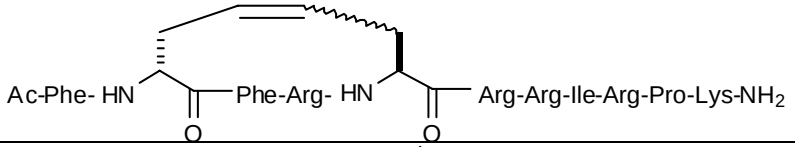
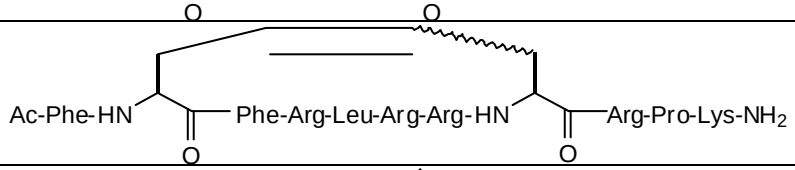
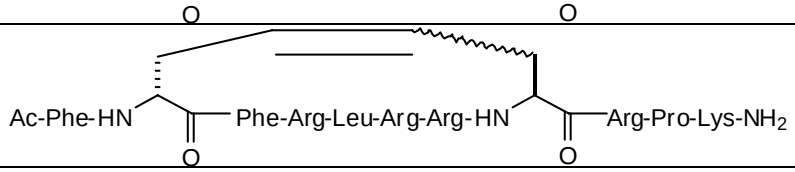
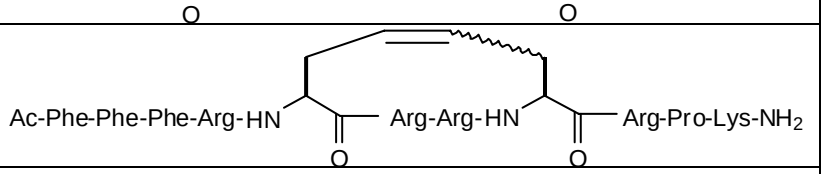
4.5[a] 4[b] 1 3 5 1 7 8 3 1 4[a]Fold decrease upon removal of the N-terminal acetyl group; [b]Numbers indicate fold decrease upon Ala substitution of the residue above the number.

A. Cyclizations involving the side chains of two D/L-AllGly residues

Cyclic arodyn analogs involving the side chains from two D/L-AllGly substituted at positions 2, 5 and/or 8 (i.e., [2,5], [2,8] and [5,8] cyclic analogs) were first designed to

examine the effects of cyclization on κ opioid receptor affinity, selectivity, and efficacy. As noted above, substitution with an Ala in position 2 in arodyn is well tolerated by κ opioid receptors without a dramatic change in κ receptor affinity and selectivity.[10] Both D- and L-AllGly were explored in this position for cyclization. Positions 5 and 8 in arodyn are two aliphatic amino acids, Leu and D-Ala, respectively. Substituting them with AllGly, a similar aliphatic group, should not affect possible hydrophobic interactions of arodyn with κ opioid receptors. Therefore, we designed cyclic analogs involving two of the three residues (2, 5 and 8) that are substituted with D/L-AllGly (Table 7.1, compounds **2-6**). In the [2,5] analogs **2** and **3**, Ile, found in Dyn A, was incorporated in position 8 rather than D-Ala so that the stereochemistry of this residue would be the same as in the other cyclic peptides. The corresponding linear analogs (Table 7.1, compounds **7-11**) were also synthesized for comparison in order to determine the effects of the changes in side chain functionalities on κ opioid receptor affinity, selectivity, and efficacy.

Table 7.1: Structures of arodyn analogs cyclized by RCM using the side chains of two L-/D-AllGly residues and their corresponding linear analogs

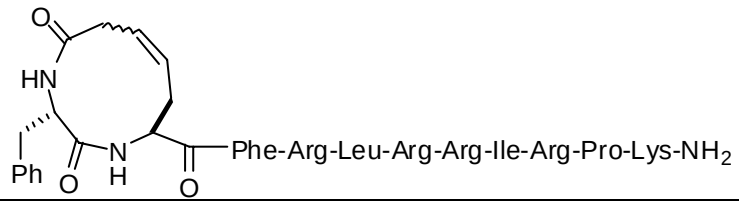
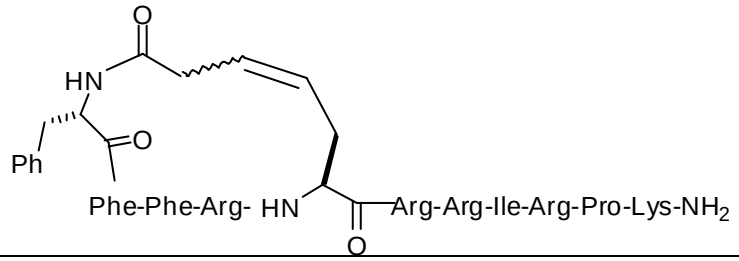
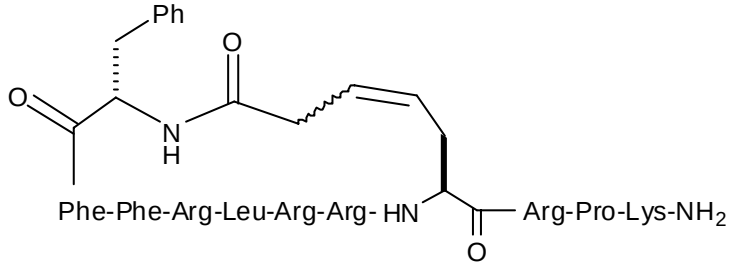
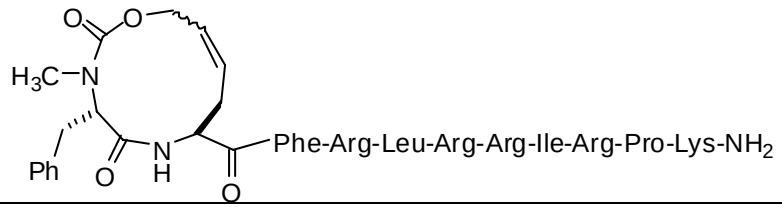
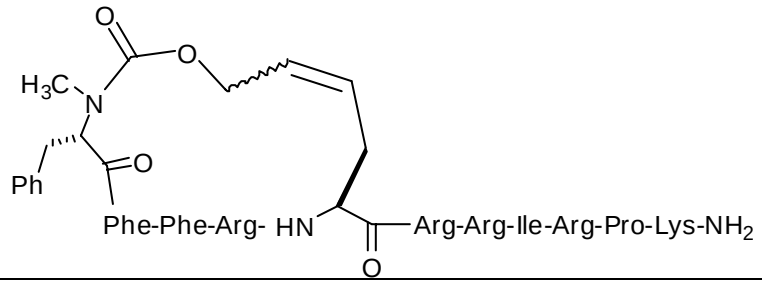
#	Compound	Structure
1	Arodyn	Ac-Phe-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Pro-Lys-NH_2
Cyclic arodyn analogs		
2	*cyclo*^{2,5}[Ala^2(-CH=CH-) Ala^5,Ile^8]	
3	*cyclo*^{2,5}[D-Ala^2(-CH=CH-) Ala^5,Ile^8]	
4	*cyclo*^{2,8}[Ala^2(-CH=CH-) Ala^8]	
5	*cyclo*^{2,8}[D-Ala^2(-CH=CH-) Ala^8]	
6	*cyclo*^{5,8}[Ala^5(-CH=CH-) Ala^8]	
Linear analogs		
7	[AllGly^{2,5},Ile^8]	Ac-Phe-AllGly-Phe-Arg-AllGly-Arg-Arg-Ile-Pro-Lys-NH_2
8	[D-AllGly^2,AllGly^5,Ile^8]	Ac-Phe-D-AllGly-Phe-Arg-AllGly-Arg-Arg-Ile-Pro-Lys-NH_2
9	[AllGly^{2,8}]	Ac-Phe-AllGly-Phe-Arg-Leu-Arg-Arg-AllGly-Pro-Lys-NH_2
10	[D-AllGly^2,AllGly^8]	Ac-Phe-D-AllGly-Phe-Arg-Leu-Arg-Arg-AllGly-Pro-Lys-NH_2
11	[AllGly^{5,8}]	Ac-Phe-Phe-Phe-Arg-AllGly-Arg-Arg-AllGly-Pro-Lys-NH_2

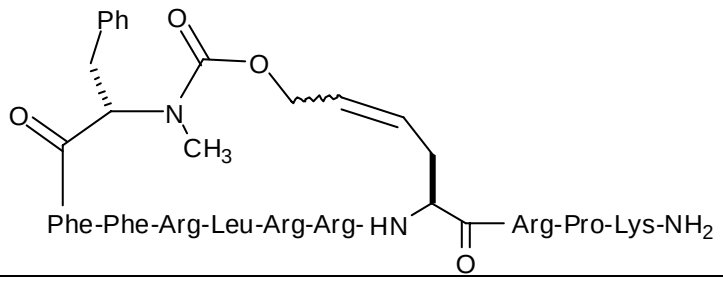
B. Cyclizations involving an N-terminal vinylacetyl/Alloc group and the side chain of an AllGly residue

The second strategy involves the so-called "N-terminus-to-side chain" cyclization. This type of cyclization could constrain the important N-terminal "message" sequence of arodyn. Arodyn is N-terminal acetylated and, as mentioned previously, capping of the N-terminal amine with an acetyl group increases κ opioid receptor affinity

by about 4-fold. Therefore, an additional carbon-carbon double bond was attached to the acetyl group (vinylacetyl) for cyclization. The cyclizations involved the N-terminal vinylacetyl group and AllGly substituted in either position 2, 5 or 8 (compounds **12-14**, Table 7.2). Unfortunately, these cyclizations were unsuccessful as only linear precursors were recovered (see below). Previously our laboratory had successfully synthesized *cyclo*^{N,5}[Trp^3,Trp^4,Glu^5]Dyn A(1-11)NH_2 (cyclodyn) by amide bond formation.[11] The failure of cyclization by RCM was probably due to the preferred conformation of the peptide. In order to cyclize successfully, the vinylacetyl group must be oriented toward the AllGly. As we discussed in Chapter 3, substitution of the N-terminal Phe by NMePhe can favor a folded structure. Furthermore, [NMePhe^1]arodyn shows higher affinity and selectivity toward κ opioid receptors.[10] Therefore, cyclizations involving NMePhe^1 analogs were designed. However, as also discussed in Chapter 3, the acyl-NMePhe analogs can undergo deletion under acidic cleavage conditions. This side reaction can be avoided by substitution of the acetyl group with an alkyloxycarbonyl group. Therefore, the allyloxycarbonyl (Alloc) group was used for the cyclizations, and the [N,2], [N,5] and [N,8] cyclizations were performed (Table 7.2, compounds **15-17**). Similarly, the linear analog (Alloc-[NMePhe^1,AllGly^5,Ile^8]arodyn, compound **18**) of the successfully synthesized cyclic peptide **16** was also prepared.

Table 7.2: Attempted cyclizations by RCM involving an N-terminal vinylacetyl/Alloc group and the side chain of an AllGly residue in either position 2, 5 or 8. Only compound **16** was successfully obtained (see text).

#	Compound	Structure
1	Arodyn	Ac-Phe-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Pro-Lys-NH_2
Cyclic arodyn analogs		
12	*cyclo*^{N,2}[$COCH_2$(CH=CH-)Ala^2, Ile^8]	
13	*cyclo*^{N,5}[$COCH_2$(CH=CH-)Ala^5, Ile^8]	
14	*cyclo*^{N,8}[$COCH_2$(CH=CH-)Ala^8]	
15	*cyclo*^{N,2}[$COOCH_2$(CH=CH-) Ala^2,NMePhe^1,Ile^8]	
16	*cyclo*^{N,5}[$COOCH_2$(CH=CH-) Ala^5,NMePhe^1,Ile^8]	

17	*cyclo*^{N,8}[COOCH$_2$(CH=CH-) Ala^8]	

C. Cyclizations involving the side chains of Tyr(All) and AllGly residues

Phe^1 and Phe^3 contribute to κ opioid receptor affinity.[10] By using allyl-protected Tyr for RCM, we can constrain these important residues while maintaining the aromatic functionality of Phe^1 and Phe^3. Arodyn has three Phe residues in the N-terminus, and therefore serves as a good model peptide to study the scope and limitations of cyclizations involving allyl-protected Tyr.

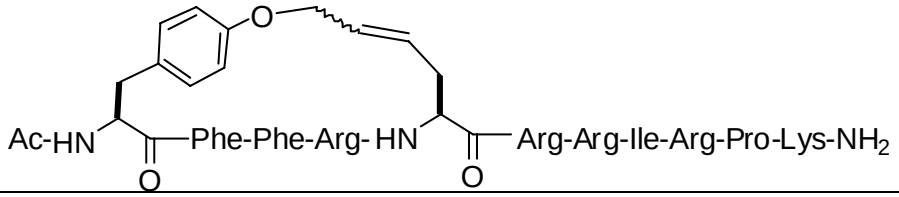
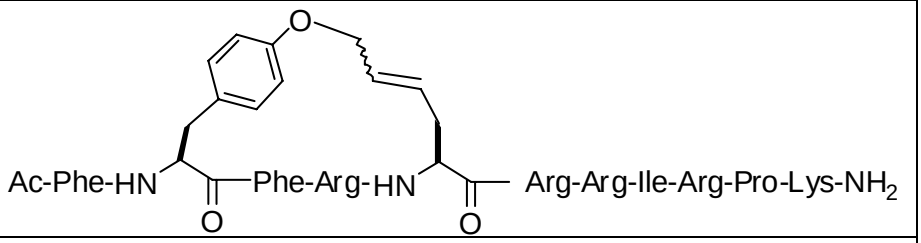
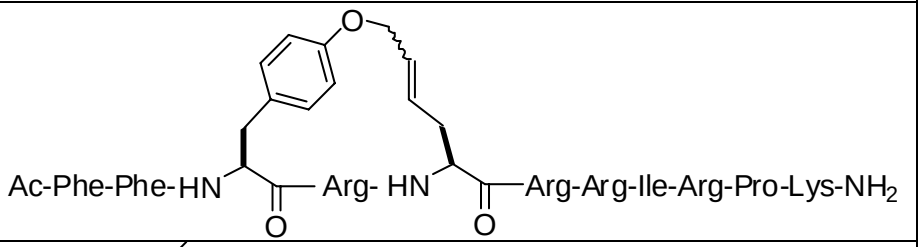
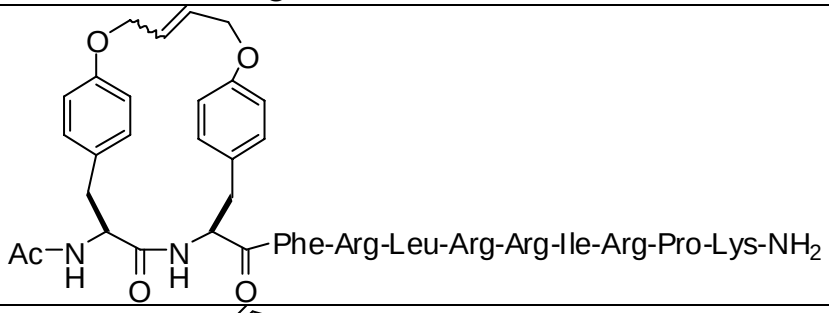
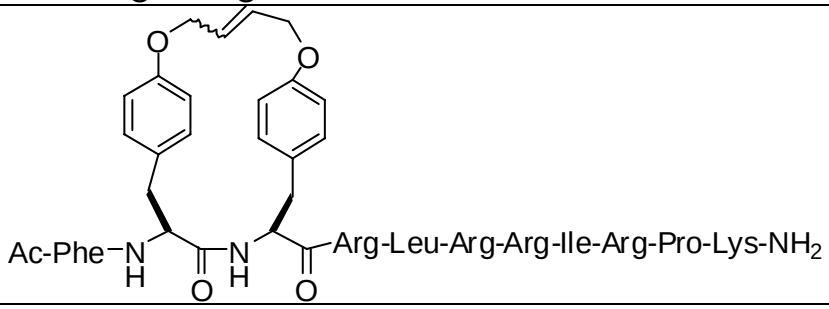
We first investigated the cyclization involving the side chains of a Tyr(All) residue and AllGly. This involved cyclization of AllGly in position 5 with Tyr(All) in one of the first three residues (Table 7.3, compounds **19-21**).

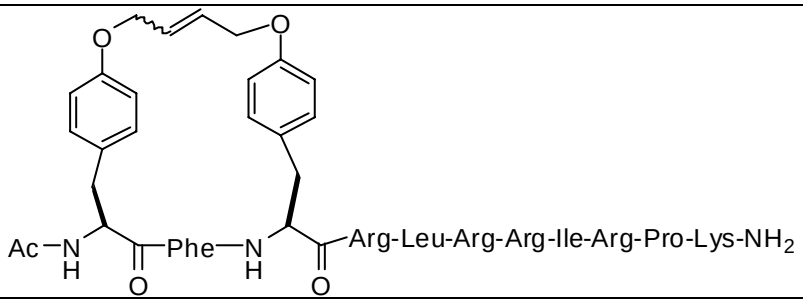
D. Cyclizations involving the side chains of two Tyr(All) residues

The last strategy for cyclization involved the side chains of two Tyr(All) residues. Two of the first three Phe residues in arodyn were substituted with Tyr(All), and cyclizations attempted to afford the desired cyclic peptides (compounds **22-24**, Table 7.3). In this manner, two of the three Phe residues in the N-terminus of arodyn could be constrained.

Linear compounds where each of the first three Phe residues was substituted with Try(All) were also prepared to evaluate the effects of this type of substitution on the pharmacological activities (compounds **25-27**, Table 7.3).

Table 7.3: Attempted cyclizations by RCM using the side chain(s) of Tyr(All) residue (**19**-**24**) and the linear analogs (**25**-**27**) where one of the N-terminal residues was substituted with Tyr(All). The cyclizations for **19**, **20**, and **24** were unsuccessful (see text).

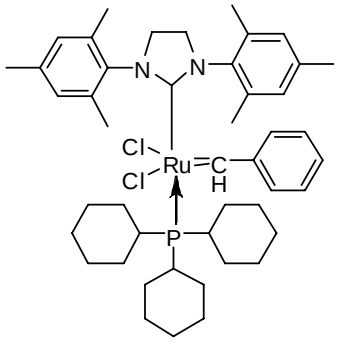
#	Compound	Structure
1	Arodyn	Ac-Phe-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Pro-Lys-NH_2
Cyclic arodyn analogs		
19	*cyclo*^{1,5}[Tyr^1(CH_2-CH=CH)Ala^5, Ile^8]	
20	*cyclo*^{2,5}[Tyr^2(CH_2-CH=CH)Ala^5, Ile^8]	
21	*cyclo*^{3,5}[Tyr^3(CH_2-CH=CH)Ala^5,Ile^8]	
22	*cyclo*^{1,2}[Tyr^1(CH_2-CH=CH-CH_2) Tyr^2,Ile^8]	
23	*cyclo*^{2,3}[Tyr^2(CH_2-CH=CH-CH_2) Tyr^3,Ile^8]	

24	*cyclo*^{1,3}[Tyr^{1}(CH_2-CH=CH-CH_2) Tyr^{3},Ile^{8}]	
25	[Tyr(All)^{1},Ile^{8}]	Ac-Tyr(All)-Phe-Phe-Arg-Leu-Arg-Arg-D-Ala-Pro-Lys-NH_2
26	[Tyr(All)^{2},Ile^{8}]	Ac-Phe-Tyr(All)-Phe-Arg-Leu-Arg-Arg-D-Ala-Pro-Lys-NH_2
27	[Tyr(All)^{3},Ile^{8}]	Ac-Phe-Phe-Tyr(All)-Arg-Leu-Arg-Arg-D-Ala-Pro-Lys-NH_2

7.2.2 Chemistry

Second-generation Grubbs' catalyst (Figure 7.2) was used to cyclize the peptides on the solid support. A mixture of DCM and DMF (4/1, v/v) was used as the solvent for the RCM reactions. The addition of a small amount of DMF has several advantages since DMF is compatible with both the peptide chain and the hydrophilic resin and it also allows a higher temperature to be used for the reactions.

Figure 7.2: Structure of second-generation Grubbs' catalyst.



7.2.2.1 Cyclizations involving the side chains of two D/L-AllGly

These cyclizations involved non-critical residues 2, 5 and 8 of arodyn. The position and stereochemistry of AllGly did not have much influence on the yields of the

desired cyclic peptides. Cyclizations between the side chains of AllGly with both L- and D-configurations generally gave high yields of the desired cyclic peptides (70-90%, Table 7.4). Both the *cis* and *trans* isomers were observed for all analogs. The ratio of *cis* to *trans* isomers, as determined by nuclear magnetic resonance (NMR), varied from approximately 1.2:1 to 1:4.2, depending on the position and stereochemistry of the AllGly residues (Table 7.4).

Table 7.4: Yield and *cis*/*trans* ratio of Dyn A analogs cyclized by RCM.

Entry	Compound	Products (% by HPLC)	*cis*/*trans*[a]
2	*cyclo*^{2,5}[Ala^2(-CH=CH-)Ala^5, Ile^8]	90%	1:1.7
3	*cyclo*^{2,5}[D-Ala^2(-CH=CH-)Ala^5, Ile^8]	70%	1:2.1
4	*cyclo*^{2,8}[Ala^2(-CH=CH-)Ala^8]	76%	1:3.3
5	*cyclo*^{2,8}[D-Ala^2(-CH=CH-)Ala^8]	82%	1:4.2
6	*cyclo*^{5,8}[Ala^5(-CH=CH-)Ala^8]	77%	1.2:1

[a]Ratio determined by NMR, see Table 7.6 for details.

Since the *cis* and *trans* isomers had very similar retention times (within 0.9 min) in our standard high-performance liquid chromatography (HPLC) system (5-50% of MeCN with 0.1% TFA over 45 min), a very slow gradient (0.1% change in MeCN/min) was used for purification. The two isomers of **6** could not be separated by the slow gradient of MeCN. The MeOH/water system, on the other hand, resulted in better separation, even though the peaks were much broader. Therefore, we purified these two isomers by using a slow gradient of MeOH/water (0.1% change in MeOH/min). The purified isomers were characterized by HPLC, electrospray ionization mass spectrometry (ESI-MS) and NMR (Tables 7.5 and 7.6).

Table 7.5: HPLC and MS data of purified peptides **1**-**6**, **16**, and their linear analogs.

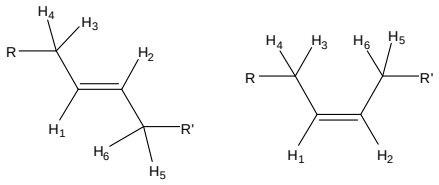
Peptides	HPLC (t_R (min)/% Purity)		ESI-MS (*m/z*)	
	System 1[a]	System 2[b]	Calculated	Observed
Cyclic peptides				
2-*cis*	15.91/100	29.60/100	$[M+4H]^{4+}$=371.5 $[M+3H]^{3+}$=495.0	$[M+4H]^{4+}$=371.4 $[M+3H]^{3+}$=494.9
2-*trans*	16.32/100	30.17/100	$[M+4H]^{4+}$=371.5 $[M+3H]^{3+}$=495.0	$[M+4H]^{4+}$=371.4 $[M+3H]^{3+}$=494.9
3-*cis*	15.91/100	30.72/100	$[M+4H]^{4+}$=371.5 $[M+3H]^{3+}$=495.0	$[M+4H]^{4+}$=371.5 $[M+3H]^{3+}$=494.9
3-*trans*	16.02/100	32.44/100	$[M+4H]^{4+}$=371.5 $[M+3H]^{3+}$=495.0	$[M+4H]^{4+}$=371.5 $[M+3H]^{3+}$=494.9
4-*cis*	17.07/100	31.36/100	$[M+4H]^{4+}$=371.5 $[M+3H]^{3+}$=495.0 $[M+5H]^{5+}$=297.4	$[M+4H]^{4+}$=371.5 $[M+3H]^{3+}$=495.0 $[M+5H]^{5+}$=297.4
4-*trans*	16.18/100	29.17/100	$[M+4H]^{4+}$=371.5 $[M+3H]^{3+}$=495.0 $[M+5H]^{5+}$=297.4	$[M+4H]^{4+}$=371.5 $[M+3H]^{3+}$=495.0 $[M+5H]^{5+}$=297.4
5-*trans*	16.10/100	29.56/100	$[M+4H]^{4+}$=371.5 $[M+3H]^{3+}$=495.0 $[M+5H]^{5+}$=297.4	$[M+4H]^{4+}$=371.5 $[M+3H]^{3+}$=495.0 $[M+5H]^{5+}$=297.4
6-*cis*	20.60/100	34.77/100	$[M+4H]^{4+}$=380.0 $[M+3H]^{3+}$=506.3	$[M+4H]^{4+}$=379.9 $[M+3H]^{3+}$=506.3
6-*trans*	20.76/100	35.38/100	$[M+4H]^{4+}$=380.0 $[M+3H]^{3+}$=506.3	$[M+4H]^{4+}$=379.9 $[M+3H]^{3+}$=506.3
16-*trans*	25.81/100	23.52/100[c]	$[M+4H]^{4+}$=398.0 $[M+3H]^{3+}$=530.3	$[M+4H]^{4+}$=398.0 $[M+3H]^{3+}$=530.3
Linear analogs				
7	19.09/100	34.05/100	$[M+4H]^{4+}$=378.5 $[M+3H]^{3+}$=504.3 $[M+5H]^{5+}$=303.0	$[M+4H]^{4+}$=378.5 $[M+3H]^{3+}$=504.3 $[M+5H]^{5+}$=303.0
8	20.03/99.1	35.51/100	$[M+4H]^{4+}$=378.5 $[M+3H]^{3+}$=504.3	$[M+4H]^{4+}$=378.5 $[M+3H]^{3+}$=504.3
9	18.78/98.9	33.25/99.4	$[M+4H]^{4+}$=378.5 $[M+3H]^{3+}$=504.3	$[M+4H]^{4+}$=378.5 $[M+3H]^{3+}$=504.3
10	19.70/99.2	34.85/99.7	$[M+4H]^{4+}$=378.5 $[M+3H]^{3+}$=504.3	$[M+4H]^{4+}$=378.5 $[M+3H]^{3+}$=504.3
11	23.03/100	21.27/100[c]	$[M+4H]^{4+}$=387.0 $[M+3H]^{3+}$=515.6	$[M+4H]^{4+}$=387.0 $[M+3H]^{3+}$=515.6
18	30.29/100	33.63/100[c]	$[M+4H]^{4+}$=405.0 $[M+3H]^{3+}$=539.7	$[M+4H]^{4+}$=405.0 $[M+3H]^{3+}$=539.7

[a]System 1: 5-50% Solvent B over 45 min (Solvent A= H_2O containing 0.1% TFA; Solvent B= MeCN containing 0.1% TFA); [b]System 2: 5-50% Solvent B over 45 min (Solvent A= H_2O containing 0.1% TFA; Solvent B= MeOH containing 0.1% TFA); [c]25-70% Solvent B over 45 min

The NMR J-couplings and chemical shifts were used to distinguish between the *cis* and *trans* isomers.[12-14] The splitting patterns of the vinyl protons are very different for the *cis* and *trans* isomers as they are coupled to adjacent methylene protons with different coupling constants. For the *trans* isomer, the coupling constants between the two vinyl protons (J_{12}) are around 15 Hz, while the coupling constants between the vinyl protons and their corresponding adjacent methylene protons (J_{13}, J_{14}, J_{25} and J_{26}) are around 7-8 Hz. Because of the coupling constants, the peaks for the *trans* isomers are generally split into 5 peaks in the spectrum (Figure 7.3). The approximate ratio for the five peaks is 1:2:2:2:1. For the *cis* isomer, the coupling constants between the two vinyl protons (J_{12}) are around 10 Hz, while the coupling constants between the vinyl protons and adjacent methylene protons are around 10 and 2 Hz. Because of the broad linewidth, only three peaks are generally observed for the *cis* isomers in a ratio of 1:2:1 (Figure 7.3). The chemical shifts of the two vinyl protons in the cyclic peptides are generally between 5.0 and 5.4 ppm (Table 7.6). Generally, the two vinyl protons in the *cis* isomer are slightly more shielded (upfield) than in the *trans* isomers (Figure 7.3 and Table 7.6).

The 1D ^{1}H-NMR for **4**-*trans* and **5**-*trans* are more complex. For **4**-*trans*, the two peaks for the two vinyl protons are very broad without obvious splitting, and therefore the coupling constants were difficult to determine. Peptide **5**-*trans* has better splitting. The coupling constants between the two vinyl protons were determined to be 15 Hz (Table 7.6). The spectra of **4**-*trans* and **5**-*trans* are very similar to each other. Based on this, we assigned the isomers with the smaller retention time as the *trans* isomer.

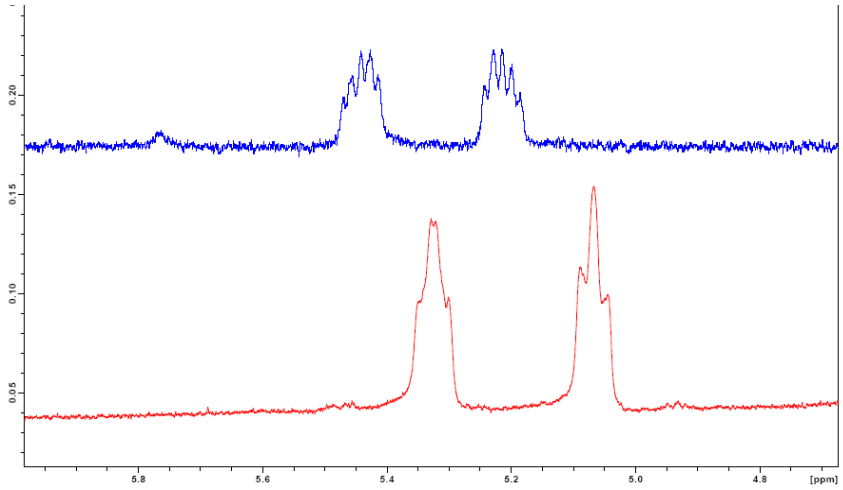
Table 7.6: ^{1}H-NMR data of cyclic peptides **1-6** and **16**



Peptides	Chemical shifts (δ) of vinyl protons	*J* (Hz)
2-*cis*	H_1 = 5.07	J_{12} = 10.9
	H_2 = 5.33	J_{21} = 10.7
2-*trans*	H_1 = 5.21	J_{12} = 14.3
	H_2 = 5.44	J_{21} = 14.8
3-*cis*	H_1 = 5.04	J_{12} = 10.3
	H_2 = 5.22	J_{21} = 11.1
3-*trans*	H_1 = 5.08	J_{12} = 14.6
	H_2 = 5.35	J_{21} = 14.3
4-*trans*[a]	H_1 = 5.14	J_{12} = 15[b]
	H_2 = 5.21	J_{21} = 15[b]
5-*trans*[a]	H_1 = 5.17	J_{12} = 14.7
	H_2 = 5.23	J_{21} = 15.5
6-*cis*	H_1 = 5.07	J_{12} = 10.6
	H_2 = 5.36	J_{21} = 11.2
6-*trans*	H_1 = 5.13	J_{12} = 14.5
	H_2 = 5.39	J_{21} = 14.6
16-*trans*	H_1 = 5.47	J_{12} = 15.5[c]
	H_2 = 5.60	J_{21} = 15.7[c]

[a]The 1D ^{1}H-NMR for **4**-*cis* and **5**-*cis* were not evaluated because of low yields of the purified peptide. [b]*J* values estimated based on the similarity between **4**-*trans* and **5**-*trans;* [c]*J* values determined after decoupling vinyl protons from adjacent methylene protons. See Experimental Section and Appendix 4 for details.

Figure 7.3: Chemical shifts and splitting patterns of the two vinyl protons of the *cis* (bottom) and *trans* (top) isomers of **2**.



7.2.2.2 Cyclizations involving an N-terminal vinylacetyl/Alloc group and the side chain of an AllGly residue

Cyclizations involving an N-terminal vinylacetyl group and AllGly substituted in either position 2, 5, or 8 of arodyn (compounds **12-14**) were unsuccessful. Only the linear precursors were recovered, and no cyclization products were observed by either HPLC or ESI-MS following 2 days reaction. Previously we successfully synthesized *cyclo*^{N,5}[Trp^3,Trp^4,Glu^5]Dyn A(1-11)NH_2.[11] This compound has similar structure to **13**. The cyclization requires groups in close proximity, and, the orientation of groups, especially for RCM, may be important. Therefore, the failure of the cyclization of **13** may be due to an extended structure in the N-terminal acyl group.

Cyclizations involving an N-terminal Alloc group and the side chain of AllGly were more complicated. The [N,2] cyclization (**15**) was also unsuccessful. The desired cyclic peptide has a medium size ring (11 atoms), and therefore has ring strain inherent in the cyclic product. Similar results have been reported in the literature.[2, 4] The [N,8]

cyclization (**17**) was unsuccessful as well, probably due to the large distance between the two double bonds. The only successful reaction was the [N,5] cyclization (**16**), with a 40% yield. Only the *trans* isomer was observed for the cyclic product based on NMR. The 1D ^{1}H-NMR spectrums for the vinyl protons of **16** are very complex. After double decoupling of the adjacent methylene protons, the two vinyl protons show an AB pattern (see Appendix 4). The chemical shifts and the coupling constants were then determined accordingly (Table 7.6).[12] The presence of an N-methyl group in Phe^{1} probably favors a folded conformation, which facilitates the cyclization reaction. The HPLC and MS data are shown in Table 7.5.

7.2.2.3 Cyclizations involving the side chains of a Tyr(All) and an AllGly residue

By this method of cyclization, the important aromatic residues (Phe^{1} and Phe^{3}) of arodyn could be constrained. However, the cyclizations were unsuccessful for the [1,5] and [2,5] cyclic peptides (compounds **19** and **20**, respectively), and only the des-OAll Tyr products were observed. This is a new side reaction that has not been reported before. More details on the mechanism of this side reaction will be discussed later in section 7.2.2.5.

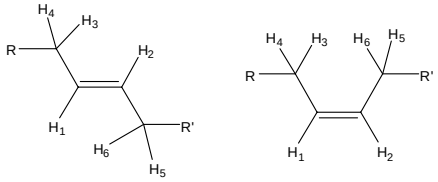
For the [3,5] cyclic peptide (**21**), both the cyclic and des-OAll products were observed, with the cyclic peptide constituting 50% of the products based on the HPLC chromatogram. Only the *trans* isomer was observed, with coupling constants between the two vinyl protons around 16 Hz (Table 7.8). This peptide was purified by HPLC and the analytical data are shown in Tables 7.7 and 7.8. Structural comparisons of the [3,5] cyclic peptide (**21**), the des-OAll product, and arodyn are shown in Figure 7.4.

Table 7.7: HPLC and MS data of purified peptides **21-23** and linear analogs (**25-27**) substituted with a Tyr(All) in the N-terminus of arodyn.

#	HPLC (t_R (min)/% Purity)		ESI-MS (*m/z*)	
	System 1[a]	System 2[b]	Calculated	Observed
21	20.94/100	38.68/100	$[M+4H]^{4+}$=398.0 $[M+3H]^{3+}$=530.3	$[M+4H]^{4+}$=398.0 $[M+3H]^{3+}$=530.3
22	27.11/100	23.13/100[c]	$[M+4H]^{4+}$=416.2 $[M+3H]^{3+}$=554.7	$[M+4H]^{4+}$=416.2 $[M+3H]^{3+}$=554.7
23	24.87/100	44.44/100	$[M+3H]^{3+}$=554.7 $[M+2H]^{2+}$=831.0	$[M+3H]^{3+}$=554.7 $[M+2H]^{2+}$=831.0
25	26.74/100	31.05/100[c]	$[M+4H]^{4+}$=409.0 $[M+3H]^{3+}$=545.0	$[M+4H]^{4+}$=409.0 $[M+3H]^{3+}$=545.0
26	27.57/100	31.53/100[c]	$[M+4H]^{4+}$=409.0 $[M+3H]^{3+}$=545.0	$[M+4H]^{4+}$=409.0 $[M+3H]^{3+}$=545.0
27	27.57/100	30.29/100[c]	$[M+4H]^{4+}$=409.0 $[M+3H]^{3+}$=545.0	$[M+4H]^{4+}$=409.0 $[M+3H]^{3+}$=545.0

[a]System 1: 5-50% Solvent B over 45 min at 1 mL/min (Solvent A= H_2O containing 0.1% TFA; Solvent B= MeCN containing 0.1% TFA); [b]System 2: 5-50% Solvent B over 45 min at 1 mL/min (Solvent A= H_2O containing 0.1% TFA; Solvent B= MeOH containing 0.1% TFA); [c]25-70% Solvent B over 45 min

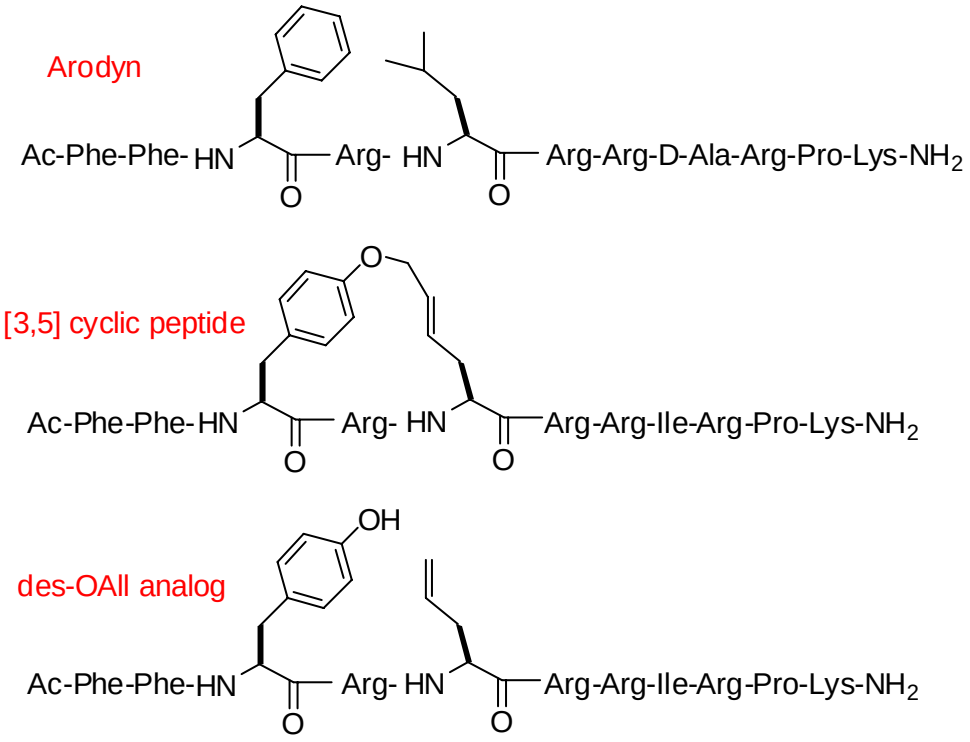
Table 7.8: ^{1}H-NMR data of cyclic peptides **21-23**



Peptides	Chemical shifts (δ) of two vinyl protons[a]	*J* (Hz)[a]
21-*trans*	H_1 = 5.51	J_{12} = 15.3
	H_2 = 5.58	J_{21} = 16.3
22-*trans*	H_1 = 5.81	J_{12} = 16.0
	H_2 = 5.86	J_{21} = 16.6
23-*trans*	H_1 = 5.81	J_{12} = 16.0
	H_2 = 5.89	J_{21} = 16.1

[a]Chemical shift and *J* values determined after decoupling vinyl protons from adjacent methylene protons. See Experimental Section and Appendix 4 for details.

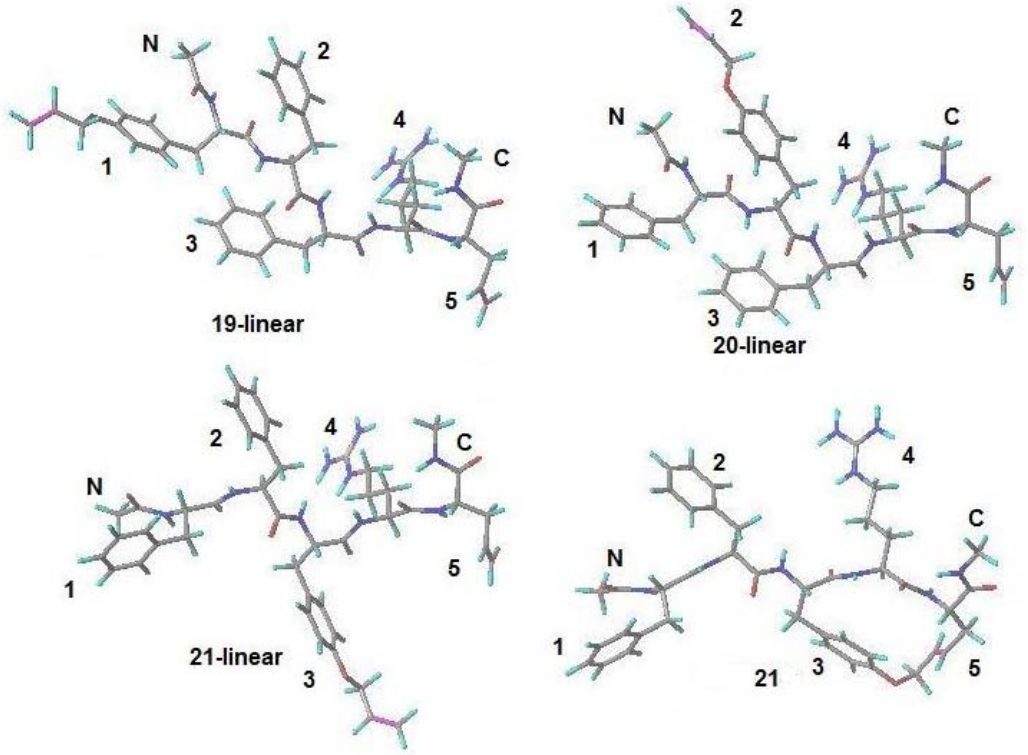
Figure 7.4: Structures of arodyn, the [3,5] cyclic peptide (**21**) and its des-OAll analog



Preliminary modeling on the linear and cyclic peptides was performed using the Biopolymer module in Sybyl 7.0 to examine possible reasons for the differences in the success of the cyclizations. The structures were energy minimized. The two double bonds of the 1,5-substituted (**19**-linear, left top) and the 2,5-substituted (**20**-linear, right top) linear peptides are far away from each other (22.0 and 17.4 angstroms (Å), respectively, Figure 7.3 and Table 7.9). In order to form the cyclic peptides, the linear peptides must undergo huge conformational changes that may imply energy barrier to get the probably needed conformation. In this situation, the side reaction involving the deletion of the allyl group from Tyr(All) dominated, and therefore no desired cyclic peptide was formed. The two double bonds of the 3,5-substituted linear analog (**21**-linear, left bottom) were closer

to each other (10.0 Å, Table 7.9). Also the cyclic peptide **21** (right bottom) did not appear to have much ring strain. Therefore the cyclic peptide was successfully obtained.

Figure 7.5: Molecular modeling of the linear precursors of **19**-**21** and the cyclic peptide **21**[a]



[a]N indicates N-terminus, 1-5 indicate the positions of each amino acid in the peptides, while C designates the rest of the C-terminal sequence; for simplicity, it was replaced by an N-methyl group in the modeling. The double bonds are shown in magenta.

Table 7.9: Distances between the two double bonds measured between the two internal carbons of the double bonds in the linear peptides of **19**-**24**

#	Compound	Distance between two C=Cs (Å)
19	*cyclo*^{1,5}[Tyr^{1}(CH_2CH=CH)-Ala^{5}, Ile^{8}]	22.0
20	*cyclo*^{2,5}[Tyr^{2}(CH_2CH=CH)-Ala^{5}, Ile^{8}]	17.4
21	*cyclo*^{3,5}[Tyr^{3}(CH_2CH=CH)-Ala^{5},Ile^{8}]	10.0
22	*cyclo*^{1,2}[Tyr^{1}(CH_2CH=CH-CH_2)Tyr^{2},Ile^{8}]	4.5
23	*cyclo*^{2,3}[Tyr^{2}(CH_2-CH=CH-CH_2)Tyr^{3},Ile^{8}]	11.9
24	*cyclo*^{1,3}[Tyr^{1}(CH_2-CH=CH-CH_2)Tyr^{3},Ile^{8}]	6.2

7.2.2.4 Cyclizations involving the side chains of two Tyr(All) residues

For the [1,2] and [2,3] cyclic peptides (compounds **22** and **23**) involving two adjacent Tyr(All) residues, we obtained the desired cyclic peptides, although all three possible des-OAll products were also observed with different yields (Table 7.10). Based on NMR, only the *trans* isomer was observed in these two cyclic peptides. The purified peptides were characterized by HPLC, MS and NMR (Tables 7.7 and 7.8).

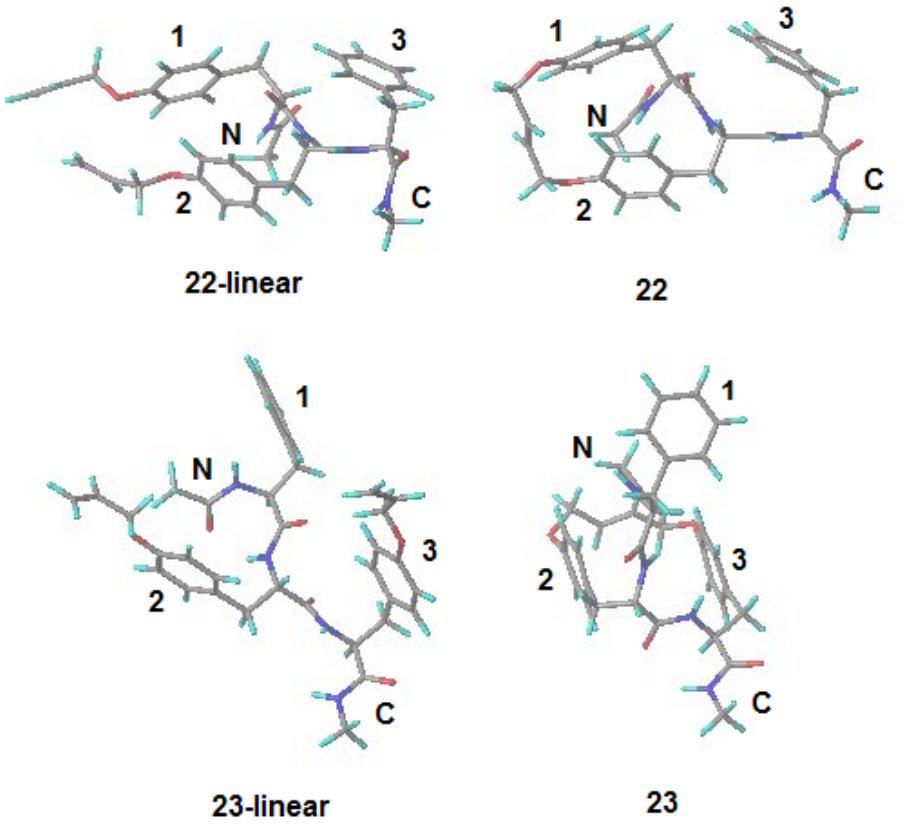
Table 7.10: Ratios of different products obtained during the synthesis of **22-24**

Desired cyclic peptide	Peak #	HPLC t_R (min)[a]	Area %	ESI-MS ($[M+3H]^+$)	Identification[b]
22					
	1	18.5	8.5	537.3	Des-OAll 1 & 2
	2	23.3	15.5	550.7	Des-OAll 1 or 2
	3	23.7	20.3	550.7	Des-OAll 1 or 2
	4	24.9	38.0	554.7	Cyclic peptide **22**
	5	28.8	17.7	561.7	Linear precursor
23					
	1	18.9	13.8	537.3	Des-OAll 1 & 2
	2	23.0	5.0	550.7	Des-OAll 1 or 2
	3	24.2	5.1	550.7	Des-OAll 1 or 2
	4	24.7	63.9	554.7	Cyclic peptide **22**
	5	27.6	9.2	561.7	Linear precursor
24					
	1	18.7	26.3	537.3	Des-OAll 1 & 2
	2	23.4	15.5	550.7	Des-OAll 1 or 2
	3	24.5	13.0	550.7	Des-OAll 1 or 2
	4	28.5	15.7	561.7	Linear precursor

[a]HPLC conditions: 50-90% Solvent B over 45 min at 1 mL/min (Solvent A= H_2O; Solvent B= MeOH, both containing 0.1% TFA); [b]Based on ESI-MS.

In preliminary molecular modeling the two double bonds in compounds **22** and **23** are relatively close (4.5 and 11.9 Å, respectively, Table 7.9 and Figure 7.6), and the desired cyclic peptides do not have strained structures; therefore the cyclizations were successful.

Figure 7.6: Molecular modeling of **22** ([1,2] cyclic peptide), **23** ([2,3] cyclic peptide) and their linear precursors

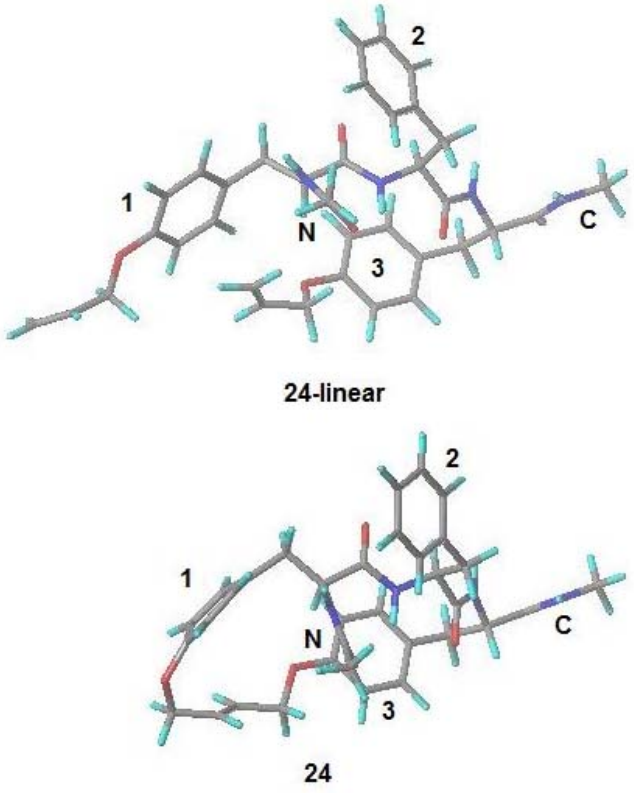


[a]N indicates N-terminus, 1-3 indicate the positions of each amino acid in the peptides, while C designates the rest of the C-terminal sequence; for simplicity, it was replaced by an N-methyl group in the modeling.

However, for [1,3] cyclic peptide (**24**), where the two Tyr(All) residues are separated by one Phe, no desired cyclic peptide was observed by ESI-MS. In contrast, the des-OAll products were observed as major products (Table 7.10). We then did similar molecular modeling of the structures of the [1,3] cyclic peptide (**24**) and its linear precursor (Figure 7.7). The two double bonds of the 1,3-substituted linear peptide (top) were observed in relatively close proximity to each other (6.2 Å, Table 7.9). But in the desired cyclic peptide (bottom), the ring system was very strained and the aromatic ring in residue 1 was not planar but distorted. This suggested that the cyclization reaction

would require high activation energy. Therefore, the side reaction involving the deletion of the allyl group from Tyr predominated and no desired cyclic peptide was obtained.

Figure 7.7: Molecular modeling of **24** ([1,3] cyclic peptide, bottom) and its linear precursor (top)



[a]N indicates N-terminus, 1-3 indicate the positions of each amino acid in the peptides, while C designates the rest of the C-terminal sequence; for simplicity, it was replaced by an N-methyl group in the modeling.

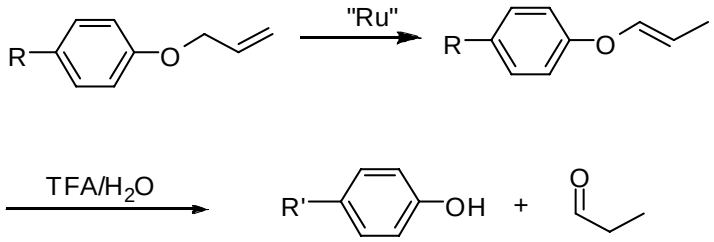
7.2.2.5 Deletion of the allyl group from Tyr(All) during the metathesis and cleavage reactions

When we attempted to make the cyclic peptide **22**, three major products other than the desired cyclic peptide were obtained (Table 7.10). These products were purified by HPLC and the molecular weights of these peptides corresponded to loss of the allyl

group from one or both of the allyl-protected Tyr residues. The deletion reaction was also observed for the other cyclization reactions involving Tyr(All).

We propose the following mechanism for the loss of the allyl group (Scheme 7.1). First, the double bond of the allyl group migrates under the metathesis conditions to form the vinyl ether intermediate, which is then hydrolyzed under acidic cleavage conditions to form the des-OAll analog. To prove this mechanism, we wanted to trap the intermediate and analyze its structure. Therefore, we examined a similar reaction in solution so that the products could be examined prior to acidic cleavage reaction.

Scheme 7.1: Proposed mechanism for the deletion of the allyl group from Tyr(All)



Fmoc-Tyr(All)-OH was treated with second generation Grubbs catalyst for two days (Scheme 7.2), and the mixture then analyzed by HPLC. In addition to the expected two cross metathesis products (Figure 7.8 A, $[M-H]^-$ = 857.3), a major product was observed (X in Figure 7.8 A). This intermediate was then purified by HPLC and its structure confirmed by ^{1}H-NMR (Figure 7.9). No des-OAll product was observed at this stage. Then the same mixture was treated with 95% TFA in water for 30 min followed by HPLC analysis, and we observed the presence of the des-allyl product Fmoc-Tyr-OH and the degradation of the intermediate X (Figure 7.8 B).

Scheme 7.2: Metathesis of Fmoc-Tyr(All)-OH

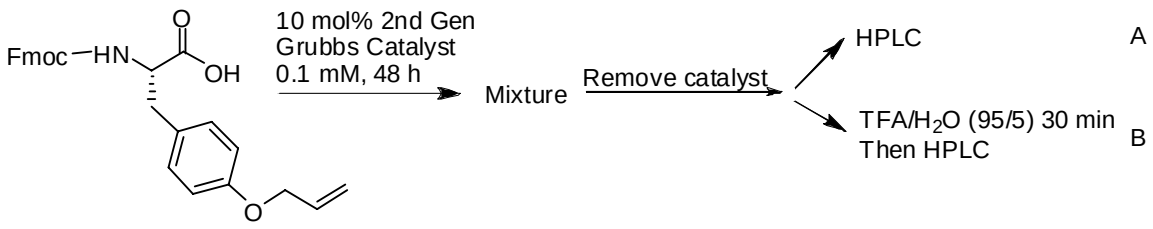
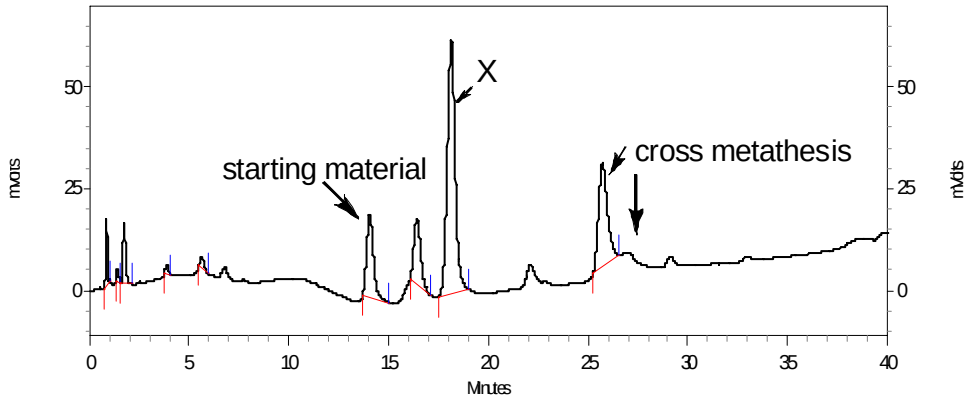
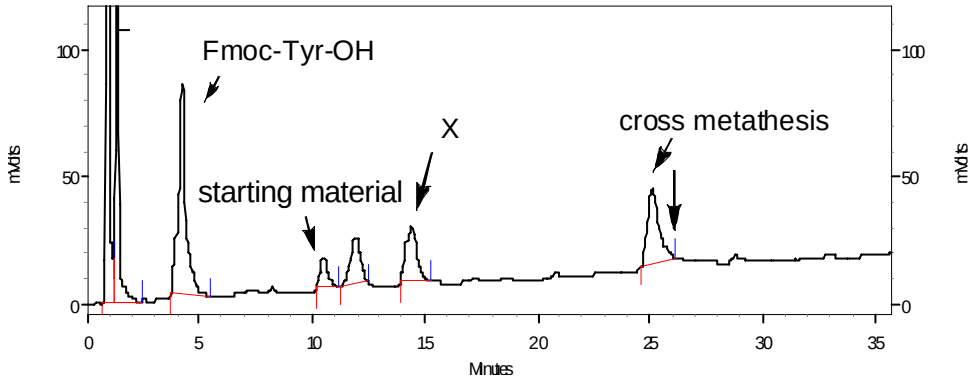


Figure 7.8: HPLC analysis[a] of the Fmoc-Tyr(All)-OH metathesis products before (A) and after (B) acidic treatment.

(A)

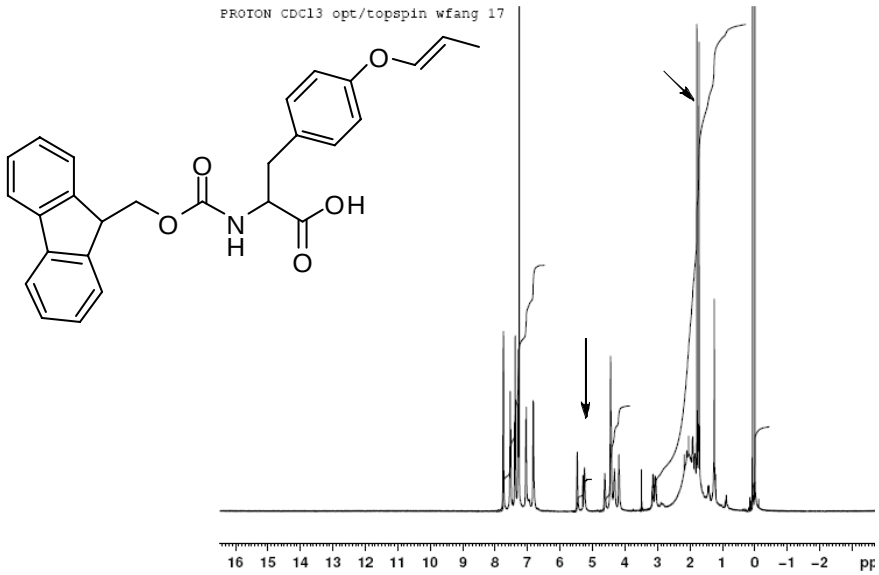


(B)



[a]HPLC conditions: 50-90% Solvent B over 45 min at 1 mL/min (Solvent A= H_2O; Solvent B= MeOH, both containing 0.1% TFA)

Figure 7.9: [1]H-NMR analysis of intermediate X[a]



[a]Arrows point to methyl and vinylether protons.

7.2.3 Pharmacology

The successfully synthesized cyclic peptides and their linear precursors were examined for their opioid receptor affinity as described previously using Chinese hamster ovary (CHO) cells stably expressing cloned opioid receptors.[15] These arodyn analogs were evaluated for their intrinsic activity at 10 μM in the adenylyl cyclase (AC) assay using cloned rat κ opioid receptors stably expressed on CHO cells.[16] The initial results are shown in Table 7.11. Additional assays are currently under way and the results will be reported as well.

Table 7.11: Pharmacological activity of cyclic arodyn analogs by RCM

Compound	K_i ± SEM (nM)		K_i ratio (κ/μ)	AC % inhibition @ 10 μM[a]
	κ	μ		
1, arodyn[b]	10.0 ± 3.0	1750 ± 130	1/175	12 ± 8
2-*cis*	130 ± 22	2310 ± 440	1/18	112 ± 4
2-*trans*	410 ± 54	>10,000	1/>24	60 ± 5
3-*cis*	200 ± 12	>10,000	1/>50	87 ± 16
3-*trans*	130 ± 20	>10,000	1/>77	38 ± 10

[a]Compared to Dyn A (1-13)NH_2 (100%). [b]From Ref.[17]

Like the cyclizations involving lactam bond formation described in Chapter 6, cyclization in the N-terminal "message" sequence of arodyn between residues 2 and 5 using RCM between two AllGly residues resulted in unfavorable κ opioid receptor binding. The *cis* isomer of **2** (*cyclo*^{2,5}[Ala^2(-CH=CH-)Ala^5,Ile^8]arodyn) shows about 13-fold lower affinity for κ opioid receptors compared to arodyn. Its affinity for μ opioid receptors also decreases by 2-fold compared with arodyn. The *trans* isomer exhibits about 3-fold lower affinity for κ opioid receptors compared with the *cis* isomer. The *trans* isomer shows negligible binding affinity for μ opioid receptors (K_i > 10 μM). The *cis* isomer of **3** (*cyclo*^{2,5}[D-Ala^2(-CH=CH-)Ala^5,Ile^8]arodyn) shows about 20-fold lower affinity for κ opioid receptors compared to arodyn. Its *trans* isomer exhibits slightly higher affinity for κ opioid receptors compared with the *cis* isomer. Both compounds show negligible binding affinity for μ opioid receptors (K_i > 10 μM). Compounds **4**-**6** are currently undergoing pharmacological testing.

Surprisingly, the cyclic arodyn analogs tested were partial to full agonists at κ opioid receptors in the adenylyl cyclase assay (38-112% intrinsic activity compared with Dyn A(1-13)-NH_2, Table 7.11). This is very interesting since *cyclo*[Asp^2,Dap^5,Ile^8]arodyn described in Chapter 6 does not show agonist activity. The

differences probably arise from the different types of cyclizations having different effects on the conformations of the residues important for κ opioid receptor binding and activation.

Cyclization in the N-terminal "message" sequence of arodyn involving residues 3 and 5 using RCM between the side chains of a Tyr(All) and an AllGly residue, respectively, is also unfavorable for κ opioid receptor binding. The *trans* isomer of *cyclo*^{3,5}[Tyr^3(CH_2-CH=CH)Ala^5,Ile^8]arodyn (**21**) shows about 40-fold lower affinity (K_i = 400 ± 40 nM) compared to arodyn (**1**, K_i = 10 nM). Interestingly, cyclization in the N-terminal "message" sequence of arodyn involving residues 2 and 3 using RCM between two Tyr(All) residues turned out to be tolerated by κ opioid receptors. The *trans* isomer of *cyclo*^{2,3}[Tyr^2(CH_2-CH=CH-CH_2)Tyr^3,Ile^8]arodyn (**23**) shows only 5.5-fold lower affinity (K_i = 55 ± 4 nM) compared to arodyn (**1**, K_i = 10 nM). This is the first arodyn analog cyclized by RCM that exhibits reasonable affinity for κ opioid receptors and is a promising lead peptide. These initial cyclic arodyn analogs containing Ile in position 8, which in arodyn decreases κ opioid receptor affinity by 4-fold (K_i = 40 nM, Bennett, Murray and Aldrich, unpublished results). Thus peptide **23** exhibits only a 40% lower κ opioid receptor affinity compared to [Ile^8]arodyn. D-Ala^8 will be incorporated in position 8 of **23** to determine whether this modification will also enhance the κ opioid receptor affinity of the cyclic peptide. The pharmacological assays on the remaining compounds will be conducted shortly.

7.3 Conclusions

In conclusion, we have successfully applied RCM to the synthesis of cyclic arodyn analogs utilizing different approaches. Cyclization by RCM has some advantages over traditional approaches such as amide bond or disulfide bond formation. By using the side chains of D/L-AllGly for cyclization, the functionalities of the lipophilic side chains of Leu and Ile can be maintained. By using Tyr(All) for cyclization, important aromatic residues can be constrained without loss of functionality.

Cyclizations involved the side chains of two AllGly residues in the non-critical 2, 5 and 8 positions of arodyn generally gave high yields of the desired cyclic peptides. Both isomers were observed in all the cases described here. Cyclizations involving an N-terminal vinylacetyl group and AllGly substituted in position 2, 5, or 8 of arodyn were unsuccessful, probably due to an extended conformation resulting in too large a distance between the two double bonds. Incorporation of an N-methyl group in Phe^1 could favor a folded structure in the N-terminus of the peptide. The cyclization involving the N-terminal Alloc group and the side chain of AllGly in position 5 was successful; only the *trans* isomer of the cyclic peptide was obtained. The [N,2] and [N,8] cyclizations were unsuccessful, probably due to ring strain (11-membered ring) inherent in the cyclic product in the first case and the long distance between the two double bonds in the second case.

By using Tyr(All) for cyclization, the important aromatic residues of arodyn could be constrained without loss of the aromatic functionality. However, the cyclization is dependent on the linear and cyclic peptide sequences and conformations. For the cyclization to be successful, the two double bonds in the linear peptide must be in close

proximity and oriented properly. Also the product should not have significant ring strain that would require a high activation energy for cyclization. Interestingly, for the successfully synthesized peptides, only the *trans* isomers were obtained as confirmed by NMR.

A side reaction involving the deletion of the allyl group from Tyr competes with the cyclization reactions involving Tyr(All). A mechanism for this side reaction has been proposed and studied using Fmoc-Tyr(All)-OH. The double bond of the allyl group migrates under the metathesis conditions to form the vinyl ether intermediate, which is then hydrolyzed under acidic cleavage conditions to form the des-OAll analog.

One cyclic analog of arodyn involving cyclization between two Tyr(All) (*cyclo*^{2,3}[Tyr^2(CH_2-CH=CH-CH_2)Tyr^3]arodyn, **23**) retains reasonable affinity (K_i = 55 nM) for κ opioid receptors. The loss (5.5-fold) of affinity may be mainly due to the substitution of Ile for D-Ala^8. Further analogs will be synthesized to increase the affinity for κ opioid receptors.

7.4 Experimental section

7.4.1 Materials

Fmoc-AllGly-OH and Fmoc-D-AllGly-OH were purchased from NeoMPS (San Diego, CA). Fmoc-Tyr(All)-OH was purchased from Senn Chemicals (San Diego, CA). Second generation Grubbs' catalyst and dimethyl sulfoxide-d_6 (DMSO-d_6) were purchased from Aldrich Chemical Co. (Milwaukee, WI). The sources of other materials are listed in Chapter 3.

7.4.2 Synthesis, purification and analysis of cyclic peptides by RCM

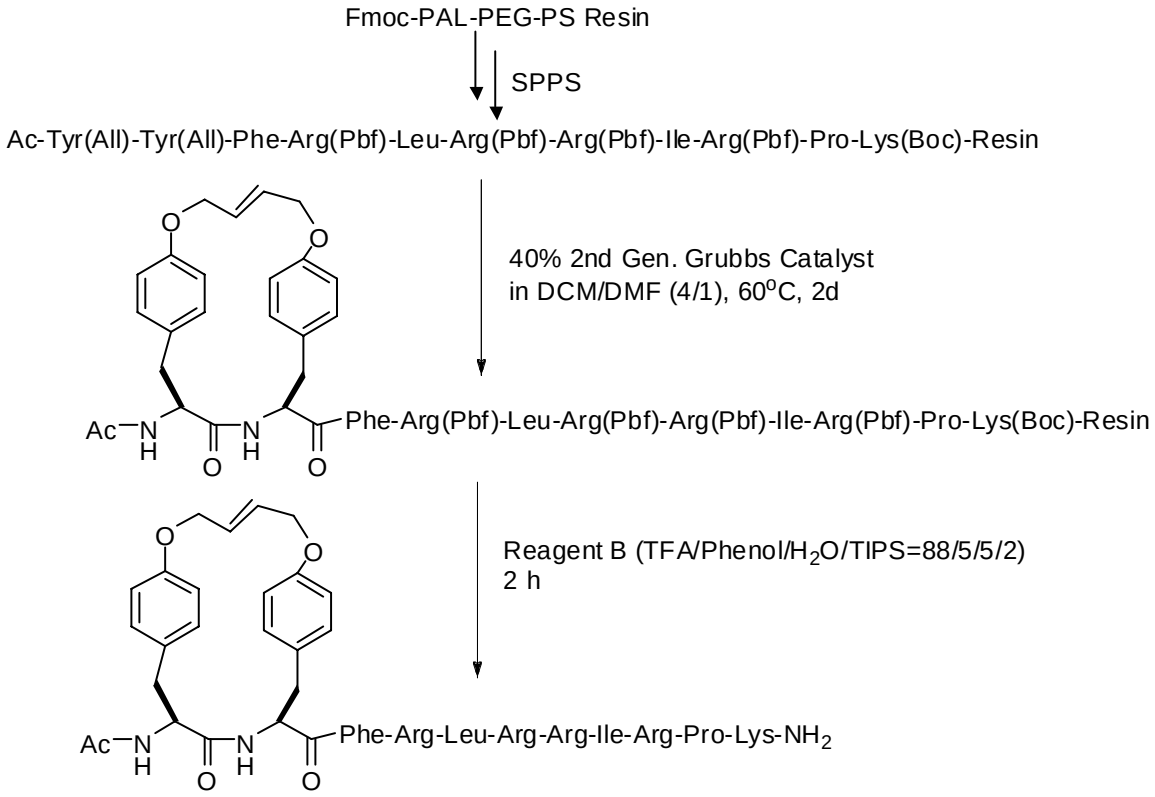
Peptide synthesis: The peptides were synthesized on the Fmoc-PAL-PEG-PS resin (PAL-PEG-PS = peptide amide linker-poly(ethylene glycol)-polystyrene, 300 mg, 0.19-0.21 mmol/g) using a CS Bio automated peptide synthesizer, except for the couplings of Fmoc-AllGly-OH and Fmoc-D-AllGly-OH (2 equiv each were used for coupling), which were performed manually. The couplings of these two amino acids were generally performed for 2 h or until completion of the reaction as determined by the ninhydrin test.[18] The synthesis of the peptide *cyclo*^{1,2}[Tyr^{1}(CH_2-CH=CH-CH_2)Tyr^{2},Ile^{8}]arodyn (**22**) is shown in Scheme 7.3 as an example. The linear precursor was first assembled on the resin by standard solid-phase peptide synthesis (SPPS) as described in Chapter 3. The resin was mixed with 40 mol% second-generation Grubbs' catalyst (3 mM, Figure 7.1) in DCM/DMF (4/1, v/v) under reflux conditions (60 ^{o}C) for 2 d (Scheme 7.3). The resin was then washed with DCM (10 X 5 mL) to remove the catalyst. Finally, the resin was shrunk by washing with methanol and dried under vacuum.

Cleavage of peptides: The peptides were cleaved from the resin by reacting with 5 mL Reagent B (88% TFA, 5% phenol, 5% water, and 2% TIPS) for 2 h as described in Chapter 3.[19]

Purification and analysis of the peptides: The successfully synthesized crude cyclic peptides were purified by preparative reversed-phase HPLC. The purification conditions were chosen individually according to the analytical HPLC chromatogram. For the purification of **2**-**5**, a linear gradient of 10-28% aqueous MeCN containing 0.1% TFA over 3 h, at a flow rate of 20 mL/min, was used. For the purification of **6**, a linear

gradient of 33-45% aqueous MeOH containing 0.1% TFA over 2 h (0.1% MeOH/min), at a flow rate of 20 mL/min, was used. For the purification of **16** and **21-23** where there was only one isomer (*trans*), a linear gradient of 15-50% MeCN containing 0.1% TFA over 35 min was used. The linear analogs (**7-11**, **18**, and **25-27**) were also purified using a linear gradient of 15-50% MeCN containing 0.1% TFA over 35 min.

Scheme 7.3: Synthesis of cyclic peptide **22**



22

The purity of the final peptides was verified by analytical HPLC using two solvent systems. For analytical HPLC, a linear gradient of 5-50% solvent B (solvent A, aqueous 0.1% TFA and solvent B, MeCN or MeOH containing 0.1% TFA) over 45

minutes, at a flow rate of 1 mL/min, was used for the analysis. The final purity of all of the peptides in both solvent systems was greater than 98%. Molecular weights of the peptides were determined by ESI-MS (Waters, analyzer Q-TOF).

Analysis of the peptides by NMR: The configuration of the double bond of the cyclic RCM compounds was established by NMR analysis. ^{1}H NMR spectra of these compounds were performed at 25 ^{o}C on a Bruker AVANCE DRX-500 spectrometer (500.13 MHz proton frequency) equipped with a 5 mm z-gradient Cryoprobe. The pure peptides (2-5 mg) were dissolved in 0.7 mL DMSO-d_6. ^{1}H chemical shifts and coupling constants were extracted from 1D spectra. ^{1}H chemical shifts were referenced to the residual DMSO signal at 2.49 ppm. Coupling constants of the two *cis* vinyl protons are around 10 Hz, while the coupling constants of the two *trans* vinyl protons are around 15 Hz.

Time shared homonuclear decoupling was performed with a standard Bruker pulse program. This program was modified locally to perform simultaneous homonuclear decoupling at two separate frequencies. The 1D ^{1}H-NMR spectra of these cyclic analogs are attached in Appendix 4.

7.4.3 Metathesis of Fmoc-Tyr(All)-OH

Fmoc-Tyr(All)-OH (44.3 mg, 0.1 mmol) and second-generation Grubbs' catalyst (8.49 mg, 0.01 mmol, 0.1 mM) were dissolved in DCM (100 mL) and allow to reaction for 2 d under refluxing conditions. Then DCM was removed, the mixture went through C_{18} Ziptip, and washed with methanol. The methanol was then removed, and part of the mixture was run analytical HPLC in a linear gradient of 50-90% solvent B (solvent A,

water and solvent B, MeOH). Another part of the compound was treated with TFA/water (95/5) for 30 min, followed by same analytical HPLC method. The intermediate X was purified using 50-90% MeOH over 40 min on the preparative column. Fmoc-Tyr-OH was obtained by treating Fmoc-Tyr(tBu)-OH with TFA/DCM (1/1) for 2 h.

7.4.4 Molecular modeling

The structures of the linear and cyclic peptides were constructed using the amino acid templates in the Biopolymer module and were assigned random geometries. The linear peptides were energy minimized and evaluated for the distance between the two double bonds. Due to the large conformational flexibility of the linear peptides, the energy-minimized conformations obtained from random geometries were used for further analysis.

7.4.5 Pharmacological assays

Radioligand binding assays were performed using cloned rat κ and μ opioid receptors stably expressed in CHO cells as described in Chapter 4. The peptides were evaluated for their ability to inhibit the synthesis of cAMP by adenylyl cyclase as described in Chapter 4.

7.5 Bibliography

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Chapter 8. Design, Synthesis, and Biological Activities of Dynorphin A Analogs Cyclized by Ring-Closing Metathesis

*Note that the compound numbers used in this chapter are only applicable within this specific chapter.

8.1 Introduction

Clinically used analgesic agents, such as morphine and its analogs, are very potent and mainly act on μ opioid receptors. However, their use is associated with some serious side effects, such as respiratory depression, physiological and psychological dependence, and constipation.[1] Therefore considerable effort has focused on the development of κ selective opioid agonists, especially those acting in the periphery, as potential analgesics without the significant clinical side effects associated with morphine and other μ receptor selective analgesic drugs.[2, 3] Besides their roles in analgesia, κ opioid receptor agonists may also have other therapeutic applications, which include the treatment of cocaine dependence,[4] as neuroprotective and anticonvulsant agents,[5] and the treatment of HIV-1 and HIV-1 related encephalopathy.[6, 7] Kappa opioid receptor antagonists, on the other hand, may be useful for the treatment of opioid[8, 9] and cocaine[10, 11] addiction and as antidepressant agents.[12] Therefore, ligands for κ opioid receptors are very useful for studying the functions of κ opioid receptors at the molecular level, which in turn could be very important in the development of new therapeutic agents.

Dynorphin A (Dyn A, Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln), a heptadecapeptide first isolated from porcine pituitary,[13] is an endogenous ligand for κ opioid receptors and is thought to be involved in a variety of physiological functions.[14] Dyn A has an identical N-terminal tetrapeptide sequence (the "message" sequence, Tyr-Gly-Gly-Phe)[15] as most other mammalian opioid peptides and a C-terminal sequence (the "address" sequence)[15] which is unique to Dyn A. Dyn A-(1-13) and Dyn A-(1-11) exhibit similar κ opioid receptor activity to Dyn A,[15] and therefore these two shorter peptides have often served as parent structures for structure-activity

relationship (SAR) studies and for the development of analogs with improved κ affinity, selectivity, potency, and/or altered efficacy.

Like most linear peptides, Dyn A can adopt numerous conformations, and because of this, the biologically active conformation is not yet clear.[16-21] This inherent conformational flexibility may be one of the reasons that Dyn A also exhibits significant affinity for μ and δ opioid receptors, resulting in low selectivity for κ opioid receptors.

Conformational constraint by cyclization is one approach that can be used to restrict the flexibility of peptide molecules, and therefore is a valuable tool to study topographical requirements of receptors.[22-24] In addition, cyclization of peptides can provide potent and selective ligands for receptors when appropriate conformational constraints are incorporated,[24] because a well-fit pre-organized conformation decreases the entropy penalty for receptor binding.[23] Furthermore, cyclic peptides are often more stable to peptidases,[25-27] and therefore they can have improved pharmacokinetic profiles and represent promising lead compounds for further development.

Conformational constraint by cyclization has been successfully employed in the development of several potent opioid peptides. Several cyclic Dyn A analogs have been synthesized and evaluated for their biological activity.[1, 23, 28-33] Our laboratory previously reported several cyclic Dyn A analogs where either the "message" or "address" sequence[15] was constrained. *cyclo*[D-Asp^2,Dap^5]Dyn A(1-13)NH_2 (Dap = 2,3-diaminopropionic acid) exhibits high affinity for both κ and μ opioid receptors and therefore low selectivity for κ receptors.[28] *cyclo*[D-Asp^5,Dap^8]Dyn A(1-13)NH_2 shows modest affinity for κ opioid receptors compared with the linear peptide Dyn A(1-13)NH_2, but it shows increased selectivity for κ over μ and δ opioid receptors compared with Dyn

A(1-13)NH_2.[29] Cyclic Dyn A analogs were also pursued by other research groups utilizing either disulfide[30, 31] or amide[32, 33] bonds to constrain the peptides.

Our current interest is in the design and synthesis of cyclic Dyn A analogs utilizing ring-closing metathesis (RCM). RCM has emerged as a very useful method for making cyclic organic compounds as well as cyclic peptides.[34-37] Compared with peptides cyclized by amide or disulfide bond formation, there are some advantages of using RCM. The resultant carbon-carbon bond is more stable than an amide bond or disulfide bond.[36, 38] The disulfide bond can be cleaved *in vivo*.[36] Furthermore, in contrast to cyclization via amide or disulfide bond formation, in RCM side chain functionalities can be maintained by appropriate choice of the amino acid side chains for cyclization. Cyclization of peptides by RCM has been applied to the synthesis of a few analogs of the opioid peptides dermorphin and the enkephalins.[39-41] Some of the cyclic analogs showed potent activity for μ and δ opioid receptors. In contrast, there are no reports of longer opioid peptides (e.g. Dyn A analogs) cyclized through RCM. Here we describe our results for incorporating a cyclic constraint in the "message" (N-terminal) as well as "address" (C-terminal) sequences of Dyn A via RCM.

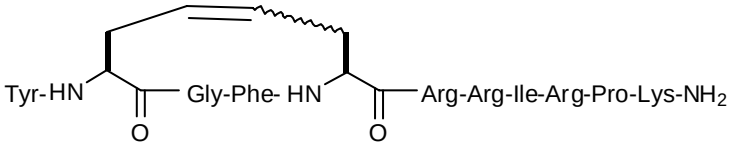
8.2 Results and discussion

8.2.1 Analog design

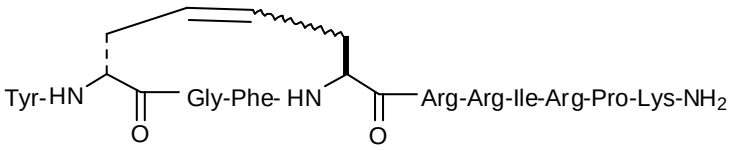
Cyclic [2,5] and [5,8] Dyn A(1-11)NH_2 analogs (Figure 8.1) were chosen to evaluate RCM for cyclizing Dyn A analogs and to examine the effects of these cyclizations on κ opioid receptor affinity, selectivity, efficacy, and potency. Substitution of a D-amino acid in position 2 for Gly in the linear peptides is well tolerated by κ opioid

receptors; however, this modification in Dyn A analogs can greatly increase μ receptor affinity, resulting in compounds that are either nonselective or selective for μ opioid receptors.[42] Substitution of this position with an L-amino acid decreases binding affinity for all three types of opioid receptors. However, κ opioid receptor is more tolerant of the L-configuration in this position than the other opioid receptors, and therefore the selectivity for κ receptor can be increased by substitution with an L-amino acid.[42] Based on these observations, in the [2,5] cyclic analogs both L- and D-allylglycine (AllGly) were introduced in position 2 to evaluate their effects on affinity, selectivity, potency, and efficacy at κ opioid receptors. Leu^5 in Dyn A is not important for opioid activity and can be used for cyclization.[43] The major differences between these cyclic peptides and the parent compound Dyn A(1-11)NH_2 are the constrained conformation plus the chiral carbon that was introduced in the second amino acid residue (Figure 8.1). Similar effects can be seen for the [5,8] cyclic analogs, where Leu^5 and Ile^8 were substituted with AllGly and then the two double bonds were cyclized through RCM (Figure 8.1).

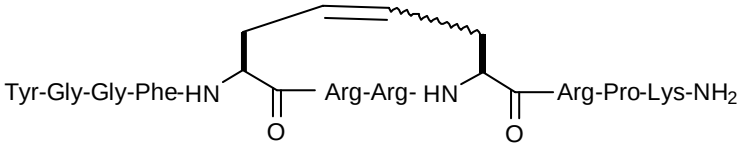
Figure 8.1: Structural comparison of *cyclo*[L/D-Ala^2(-CH=CH-)Ala^5]Dyn A (1-11)NH_2 and *cyclo*[Ala^5(-CH=CH-)Ala^8]Dyn A (1-11)NH_2 with Dyn A (1-11)NH_2



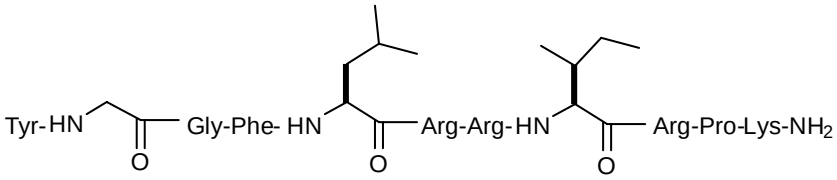
cyclo[Ala^2(-CH=CH-)Ala^5]Dyn A (1-11)NH_2



cyclo[D-Ala^2(-CH=CH-)Ala^5]Dyn A (1-11)NH_2



cyclo[Ala^5(-CH=CH-)Ala^8]Dyn A (1-11)NH_2



Dyn A (1-11)NH_2

8.2.2 Chemistry

Second generation Grubbs' catalyst (Figure 7.2) was used to cyclize the peptides on the solid support. A mixture of dichloromethane (DCM) and *N*,*N*-dimethylformamide (DMF) (4/1, v/v) was used for the RCM reaction. The addition of a small amount of DMF has several advantages since DMF is compatible with both the peptide chain and hydrophilic resin and allows a higher temperature to be used for the reaction. The position or stereochemistry of the AllGly residue did not have much influence on the yield of the desired cyclic peptides. Cyclizations between the side chains of AllGly generally gave reasonable yields of the desired cyclic peptides (56-74%, Table 8.1). Both

the *cis* and *trans* isomers were obtained for all the cyclic peptides, with the ratio of *cis* to *trans* isomers, as determined by NMR, varying from approximately 1:1.1 to 1:2.3, depending on the position and stereochemistry of the AllGly (Table 8.1).

Table 8.1: Yields and *cis*/*trans* ratios of cyclic Dyn A(1-11)NH_2 analogs by RCM

Entry	Compound	Products (% by HPLC)	*cis*/*trans*[a]
A	*cyclo*[Ala^2(-CH=CH-)Ala^5] *cis*, **1**; *trans*, **2**	56	1:1.8
B	*cyclo*[D-Ala^2(-CH=CH-)Ala^5] *cis*, **3**; *trans*, **4**	74	1:2.3
C	*cyclo*[Ala^5(-CH=CH-)Ala^8] *cis*, **5**; *trans*, **6**	63	1:1.1

[a]Configuration determined by NMR, see Table 8.3 for details.

Because the *cis* and *trans* isomers have very similar retention times (within 0.7 min) in the standard high-performance liquid chromatography (HPLC) system (5-50% of MeCN with 0.1% TFA over 45 min at 1 mL/min), a very slow gradient (0.1% change solvent B/min) was used for purification. The two isomers were successfully purified as determined by HPLC, electrospray ionization mass spectrometry (ESI-MS), and nuclear magnetic resonance (NMR) of the purified fractions (Tables 8.2 and 8.3).

Table 8.2: HPLC and ESI-MS data of purified peptides **1-6**

Peptides	HPLC (t_R (min)/% purity)[a]		ESI-MS (*m/z*)	
	System 1	System 2	Calculated	Observed
1	13.23/100	24.08/100	$[M+3H]^{3+}$=453.3 $[M+4H]^{4+}$=340.2 $[M+2H]^{2+}$=679.4	$[M+3H]^{3+}$=453.3 $[M+4H]^{4+}$=340.2 $[M+2H]^{2+}$=679.4
2	13.30/100	25.38/100	$[M+4H]^{4+}$=340.2 $[M+3H]^{3+}$=453.3 $[M+2H]^{2+}$=679.4	$[M+4H]^{4+}$=340.2 $[M+3H]^{3+}$=453.3 $[M+2H]^{2+}$=679.4
3	12.78/100	24.87/100	$[M+4H]^{4+}$=340.2 $[M+3H]^{3+}$=453.3 $[M+2H]^{2+}$=679.4	$[M+4H]^{4+}$=340.2 $[M+3H]^{3+}$=453.2 $[M+2H]^{2+}$=679.4
4	13.49/100	26.99/100	$[M+4H]^{4+}$=340.2 $[M+3H]^{3+}$=453.3 $[M+2H]^{2+}$=679.4	$[M+4H]^{4+}$=340.2 $[M+3H]^{3+}$=453.3 $[M+2H]^{2+}$=679.4
5	9.11/100	16.32/100	$[M+3H]^{3+}$=434.6 $[M+4H]^{4+}$=326.2	$[M+3H]^{3+}$=434.6 $[M+4H]^{4+}$=326.2
6	9.63/100	17.85/100	$[M+3H]^{3+}$=434.6 $[M+4H]^{4+}$=326.2	$[M+3H]^{3+}$=434.6 $[M+4H]^{4+}$=326.2

[a]Details are in the experimental section: system 1: Solvent B = MeCN; system 2: Solvent B = MeOH.

The NMR J-couplings and chemical shifts were used to distinguish between the *cis* and *trans* isomers.[44-46] The splitting patterns of the vinyl protons are very different for the *cis* and *trans* isomers as they are coupled to adjacent methylene protons with different coupling constants. For the *trans* isomer, the coupling constants between the two vinyl protons (J_{12}) are around 15 Hz, while the coupling constants between the vinyl protons and their corresponding adjacent methylene protons (J_{13}, J_{14}, J_{25} and J_{26}) are around 7-8 Hz. Because of the coupling constants, the peaks for the *trans* isomers are generally split into 5 peaks in the spectrum (Figure 8.2). The approximate ratio for the five peaks is 1:2:2:2:1. For the *cis* isomer, the coupling constants between the two vinyl protons (J_{12}) are around 10 Hz, while the coupling constants between the vinyl protons and adjacent methylene protons are around 10 and 2 Hz. Because of the broad linewidth, only three peaks are generally observed for the *cis* isomers in a ratio of 1:2:1 (Figure 8.2). The

chemical shifts of the two vinyl protons in the cyclic peptides are generally between 5.0 and 5.5 ppm (Table 7.6). Generally, the two vinyl protons in the *cis* isomer are slightly more shielded (upfield) than in the *trans* isomers (Figure 8.2 and Table 8.3).

Figure 8.2: Chemical shifts and splitting patterns of the two vinyl protons of cyclic peptides **5** (bottom) and **6** (top)

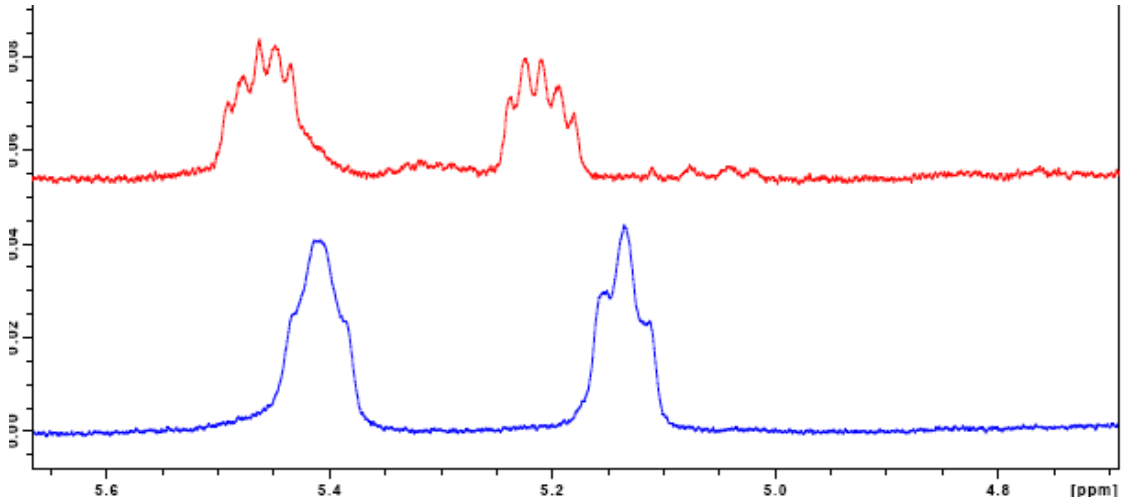
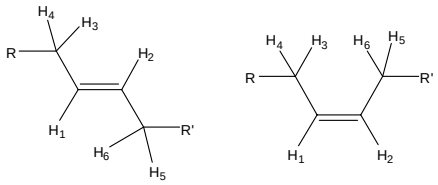


Table 8.3: ^{1}H-NMR data of cyclic peptides **1-6**



Peptides	Chemical shifts (δ) of two vinyl protons	J (Hz)[d]
1 (*cis*)	H_1 = 5.10	J_{12} = 10.4
	H_2 = 5.34	J_{21} = 12.4
2 (*trans*)	H_1 = 5.25	J_{12} = 14.9
	H_2 = 5.47	J_{21} = 13.9
3 (*cis*)	H_1 = 5.05	J_{12} = 11.4
	H_2 = 5.16	J_{21} = 7.6
4 (*trans*)	H_1 = 5.12	J_{12} = 17.4
	H_2 = 5.26	J_{21} = 15.4
5 (*cis*)	H_1 = 5.14	J_{12} = 10.5
	H_2 = 5.41	J_{21} = 12.9
6 (*trans*)	H_1 = 5.21	J_{12} = 14.9
	H_2 = 5.46	J_{21} = 13.9

8.2.3 Pharmacology

The cyclic peptides were evaluated for their binding affinity at κ, μ, and δ opioid receptors using radioligand-binding assays (Table 8.4).[29] Except for compound **1**, all of the cyclic Dyn A analogs examined exhibit reasonably high binding affinity for κ opioid receptors (K_i = 0.84 - 11 nM).

Table 8.4: Opioid receptor binding affinities of Dyn A analogs cyclized by RCM

Compound	K_i (nM)[a]			K_i ratio (κ/μ/δ)
	κ	μ	δ	
1	87.2 ± 6.9	763 ± 35	7670 ± 1030	1/8.8/88
2	9.46 ± 1.80	180 ± 10	1130 ± 98	1/19/119
3	0.84 ± 0.10	2.33 ± 0.20	9.30 ± 1.00	1/2.8/11
4	1.38 ± 0.31[b]	2.33 ± 0.22	7.17 ± 0.55	1/1.7/5.2
5	10.9 ± 1.8	93.0 ± 6.0	1210 ± 90	1/8.5/111
6	2.46 ± 0.57	36.0 ± 2.1	460 ± 51	1/15/187
Dyn A(1-11)NH_2[c]	0.57 ± 0.01	1.85 ± 0.52	6.18 ± 1.01	1/3/11

[a]Values are the mean ± SEM for three independent experiments. [b]Value is for four independent experiments. [c]From reference 47.

The [2,5] cyclizations involving an L-AllGly in position 2 decrease κ opioid receptor binding. The *trans* isomer of *cyclo*[Ala^2(-CH=CH-)Ala^5]Dyn A(1-11)NH_2 (**2**), however, has reasonable κ opioid receptor affinity (K_i = 9.5 nM) compared with Dyn A(1-11)NH_2 (Table 8.4).[47] However, the *cis* isomer **1** exhibits 10-fold lower affinity for κ receptors than the *trans* isomer **2**, presumably due to the differences in the peptides' conformations. Compared with Dyn A(1-11)NH_2, both of the two isomers **1** and **2** exhibit much lower affinity for μ and δ receptors, therefore resulting in higher κ opioid receptor selectivity for these compounds than Dyn A(1-11)NH_2 (Table 8.4). The *cis* isomer (**1**) has similar selectivity as the *trans* isomer (**2**), even though it shows about 10-fold lower affinity for κ opioid receptors. These results suggest that even thought substitution of an L-amino acid in position 2 in the cyclic peptides decreases κ receptor affinity compared with Dyn A (1-11)NH_2, the selectivity towards the other opioid receptors (μ and δ) can be increased, as these receptors are less tolerant of the introduction of an L-amino acid in position 2 compared to κ opioid receptors.

In contrast, the [2,5] cyclizations involving a D-AllGly in position 2 are well tolerated by κ opioid receptors. The two isomers of *cyclo*[D-Ala^2(-CH=CH-)Ala^5]Dyn

A(1-11)NH_2 (**3** and **4**) exhibit high affinity for κ opioid receptors (K_i = 0.84 and 1.33 nM for the *cis* and *trans* isomers, respectively, Table 8.4). However, these two compounds also exhibit high affinity for μ and δ opioid receptors, and therefore have very low selectivity for κ over these opioid receptors. These results are similar to those for *cyclo*[D-Asp^2,Dap^5]Dyn A (1-13)NH_2 and *cyclo*[D-Asp^2,Dap^5]Dyn A (1-11)NH_2.[28, 48]

Schiller and Hruby previously separately reported the synthesis of several dermorphin and enkephalin analogs utilizing RCM.[39-41] The two olefinic dicarba analogs of the enkephalin-derived cyclic peptide H-Tyr-*c*[D-Cys-Gly-Phe-L-Cys]NH_2 exhibit nanomolar affinity for both μ (2.40 nM and 0.616 nM for the *cis* and *trans* isomers, respectively) and δ (6.55 nM and 1.25 nM for the *cis* and *trans* isomers, respectively) opioid receptors.[40] As expected, these enkephalin analogs exhibit low affinity for κ opioid receptors (200 nM and 57.6 nM for the *cis* and *trans* isomers, respectively).[40] The addition of the C-terminal residues of Dyn A(1-11)NH_2 substantially increases the affinity for κ opioid receptors, while maintaining the affinity for μ opioid receptors and decreasing the affinity for δ opioid receptors. Interestingly, while the two isomers reported in that reference have different affinities for all three opioid receptors, our two compounds do not have any significant differences in opioid receptor affinities.

The cyclizations in the "address" sequence of Dyn A involving two AllGly residues substituted in positions 5 and 8 are also tolerated by κ opioid receptors. The *trans* isomer of *cyclo*[Ala^5(-CH=CH-)Ala^8]Dyn A(1-11)NH_2 (**6**) has an affinity of 2.5 nM for κ receptors, 5-fold lower compared with Dyn A(1-11)NH_2. However, this compound exhibits higher selectivity for κ over μ and δ receptors (15- and 187-fold, respectively, Table 8.4) than the linear peptide. The *cis* isomer of *cyclo*[Ala^5(-CH=CH-)Ala^8]Dyn A-

(1-11)NH_2 (**5**) shows 4.4-fold lower affinity for κ opioid receptors (K_i = 11 nM) compared with the *trans* isomer **6**. This is another case where the *cis* and *trans* isomers have different affinities. The two isomers exhibit similar selectivities for κ vs μ and δ opioid receptors (Table 8.4).

The cyclic analogs were examined for dose-dependent inhibition of adenylyl cyclase (AC) under standard assay conditions (Table 8.5).[48] All compounds inhibit AC activity in a dose-dependent manner with similar efficacy (≥ 90%) to Dyn A(1-13)NH_2. Thus the cyclizations have little or no effect on the efficacy of Dyn A(1-11)NH_2 analogs. The potencies (EC_{50}) of these cyclic analogs in the AC assays (Table 8.5) correlate with their corresponding κ opioid affinities.

Table 8.5: Potencies and efficacies for κ opioid receptor of Dyn A analogs cyclized by RCM

Compound	EC_{50} (nM)[a]	Maximum AC % inhibition[b]
1	190 ± 19	90 ± 2
2	27 ± 0.0	104 ± 4
3	0.80 ± 0.34 (4)	111 ± 6 (4)
4	0.47 ± 0.11 (4)	107 ± 4 (4)
5	23 ± 11	110 ± 10
6	8.3 ± 3.7	106 ± 6
Dyn A(1-11)NH_2	0.5	100

[a]Values are the mean ± SEM for three independent experiments except where noted; [b]Compared to Dyn A (1-13)NH_2 (100%).

The two isomers of *cyclo*[D-Ala^2(-CH=CH-)Ala^5]Dyn A(1-11)NH_2 (**3** and **4**) exhibit the highest potency with EC_{50} values (0.80 and 0.47 nM, respectively, Table 8.5) comparable to that of the parent peptide Dyn A(1-11)-NH_2. This is consistent with the results reported for *cyclo*[D-Asp^2,Dap^5]Dyn A (1-11)NH_2.[48]

For the two isomers of *cyclo*[Ala^2(-CH=CH-)Ala^5]Dyn A(1-11)NH_2 (**1** and **2**), where the configuration of position 2 is L instead of D, the potency dropped significantly (380- and 54-fold for **1** and **2**, respectively) compared with Dyn A(1-11)NH_2. Similar to the affinities for κ opioid receptors, the *trans* isomer (**2**) is about 7-fold more potent than the *cis* isomer (**1**). The two isomers of *cyclo*[Ala^5(-CH=CH-)Ala^8]Dyn A(1-11)NH_2 (**5** and **6**) show intermediate potency among these cyclic peptides, with the *trans* isomer **6** being 2.8-fold more potent than the corresponding *cis* isomer **5**.

8.3 Conclusions

In conclusion, here we report the synthesis of the first Dyn A analogs cyclized by RCM. Cyclizations in both the "message" and "address" sequences of Dyn A were explored for the successful synthesis of potent κ opioid receptor agonists. Cyclization by RCM has some advantages over traditional approaches such as amide or disulfide bond formation. The carbon-carbon double bond in these peptides retains similar lipophilicity to the side chains of Leu and Ile found in positions 5 and 8 in Dyn A. The positions and stereochemistry of the residues involved in the cyclizations influence affinity and selectivity for κ opioid receptors. The [2,5] cyclic analogs with the D-configuration in position 2 (**3** and **4**) retain high affinity for κ, μ, and δ receptors, indicating that the conformations which are adopted by these two peptides are compatible with all three opioid receptor types. These two peptides show minimal selectivity for κ over μ receptors and low selectivity for κ over δ receptors. The [5,8] cyclic analogs (**5** and **6**) show intermediate affinity for κ opioid receptors; however, their selectivity over the other opioid receptors is greater than Dyn A(1-11)NH_2. The [2,5] cyclic analogs with the L-

configuration in position 2 (**1** and **2**) show the lowest affinity for κ opioid receptors; however, these two compounds also exhibit higher selectivity for κ receptors compared with the parent peptide. In two of the three cases the configuration of the double bond has a significant influence on the opioid receptor affinity and agonist potency. Similar to Dyn A, these cyclic Dyn A analogs exhibit dose-dependent agonist activity (≥ 90% efficacy) at κ opioid receptors with potencies correlated with their affinities. These analogs represent interesting lead compounds for further characterization of conformation-activity relationships for Dyn A at κ opioid receptors.

8.4 Experimental section

8.4.1 Materials

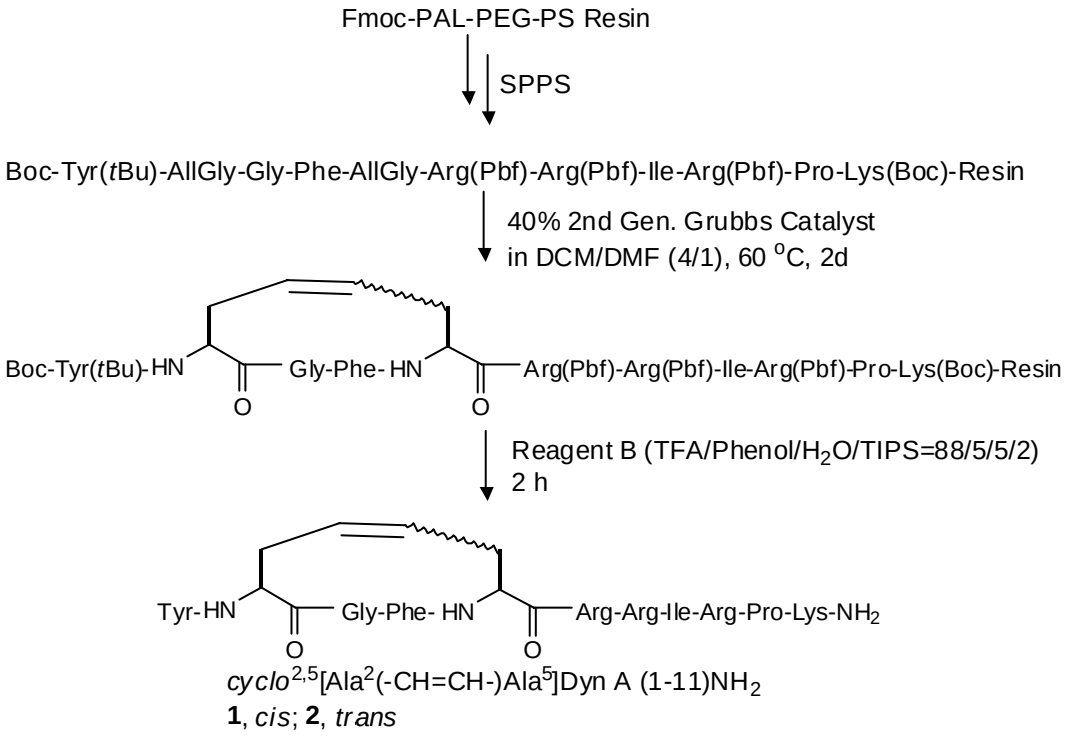
Fmoc-AllGly-OH and Fmoc-D-AllGly-OH were purchased from NeoMPS (San Diego, CA). Second generation Grubbs' catalyst and DMSO-d_6 (dimethyl sulfoxide-d_6) were purchased from Aldrich Chemical Co. (Milwaukee, WI). The sources of other materials are listed in Chapter 3.

8.4.2 Synthesis of cyclic Dyn A analogs

The synthesis of these cyclic peptides followed the procedure developed for the synthesis of cyclic arodyn analogs described in Chapter 7. The synthesis of the peptides *cyclo*[Ala^2(-CH=CH-)Ala^5]Dyn A (1-11)NH_2 (**1**, *cis*; **2**, *trans*) is shown in Scheme 8.1 as an example. The linear precursor was first assembled on the resin by standard solid-phase peptide synthesis (SPPS) as described in Chapter 3. The resin was mixed with 40 mol% second-generation Grubbs' catalyst (3 mM) in DCM/DMF (4/1, v/v) under reflux

conditions (60 ^{o}C) for 2 d (Scheme 8.1). The resin was then washed with DCM (10 X 5 mL) to remove the catalyst. Finally, the resin was shrunk by washing with methanol and dried under vacuum.

Scheme 8.1: Synthesis of cyclic peptides **1** and **2**



The crude cyclic peptides were cleaved from the resin by treating with 5 mL Reagent B (88% TFA, 5% phenol, 5% water, and 2% TIPS) for 2 h.[49] The detailed procedure is discussed in Chapter 3.

8.4.3 Analysis of cyclic Dyn A analogs

The crude peptides were analyzed by analytical reversed-phase HPLC to determine the yields and ratios of the two isomers obtained from RCM reaction. A linear

gradient of 5-50% MeCN containing 0.1% TFA over 45 minutes, at a flow rate of 1 mL/min, was used for the analysis.

The crude peptides were purified by preparative reversed-phase HPLC. For purification, a linear gradient of 5-23% aqueous MeCN containing 0.1% TFA over 3 h (0.1% MeCN/min), at a flow rate of 20 mL/min, was used. The purity of the final peptides was verified using two analytical HPLC systems and ESI-MS (Table 8.1). For analytical HPLC, a linear gradient of 5-50% solvent B (solvent A, aqueous 0.1% TFA and solvent B, MeCN or MeOH containing 0.1% TFA) over 45 minutes, at a flow rate of 1 mL/min, was used. The final purity of all peptides by both methods was greater than 98%. Molecular weights of the peptides were determined by ESI-MS (Waters, analyzer Q-TOF).

Analysis of the peptides by NMR: The configuration of the double bond of cyclic RCM compounds was established by NMR analysis. ^{1}H NMR spectra of these compounds were obtained at 25 ^{o}C on a Bruker AVANCE DRX-500 spectrometer (500.13 MHz proton frequency) equipped with a 5 mm z-gradient Cryoprobe. The pure peptides (2-5 mg) were dissolved in 0.7 mL DMSO-d_6. ^{1}H chemical shifts and coupling constants were extracted from 1D spectra. ^{1}H chemical shifts were referenced to the residual DMSO signal at 2.49 ppm. Coupling constants of the two *cis* vinyl protons are around 10 Hz, while the coupling constants of the two *trans* vinyl protons are around 15 Hz (Table 8.3). The 1D ^{1}H-NMR spectra of these cyclic analogs are attached in Appendix 5.

8.4.4 Pharmacological assays

Radioligand binding assays were performed using cloned rat κ, μ and δ opioid receptors stably expressed on CHO cells as described in Chapter 4. [^{3}H]DPDPE (*cyclo*[D-Pen^2,D-Pen^5]enkephalin) was used as the radioligand in assays for δ opioid receptors The peptides were evaluated for their ability to inhibit the synthesis of cAMP by AC as described in Chapter 4. Peptide concentration was varied from 0.1-10,000 nM in 10-fold dilutions to determine the efficacies and potencies of the peptides.

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Chapter 9. Summary, Conclusions and Future Work

The main objective of this dissertation research was to develop potent and selective peptide ligands for kappa (κ) opioid receptors and explore the influence of constraining the conformation on opioid receptor affinity and activity, based on two lead compounds arodyn and Dyn A.

Previous results indicate that the potent and highly selective κ opioid receptor antagonist [NMePhe^1]arodyn is unstable under the acidic cleavage conditions.[1] We investigated the cleavage conditions by using different cleavage cocktails as well as varying the cleavage time at 4 ^oC (Chapter 3). While alternative cleavage conditions increased the yields of the full-length peptides, the deletion reaction still occurred, decreasing the yield of the desired peptides. Substitution of the N-terminal acetyl group with a different heteroatom-containing group (for example, a methoxycarbonyl group or an amino acid) was found to prevent the side reaction (Chapter 3). On the other hand, other acyl groups, including those with bulky substituents, are not effective at preventing the deletion reaction.

Pharmacological evaluation shows that a methoxycarbonyl group can be substituted for the acetyl group in [NMePhe^1]arodyn without loss of κ opioid receptor affinity (Chapter 4). [CH_3OCO-NMePhe^1]arodyn has similar κ opioid receptor affinity as [NMePhe^1]arodyn and retains high κ opioid receptor selectivity. For other substituents (i.e. $C_6H_5OCH_2CO$, Gly, and Ac-Gly), the affinity and/or selectivity for κ receptors decreased compared to the parent peptides. The N-terminal methoxycarbonyl analogs where Phe^3 is replaced with other aromatic or non-aromatic amino acids generally retain similar affinity for κ opioid receptors as [CH_3OCO-NMePhe^1]arodyn. The selectivity of these methoxycarbonyl analogs for κ over μ opioid receptors, however, is generally lower

than [CH_3OCO-NMePhe^1]arodyn. [CH_3OCO-NMePhe^1]arodyn reverses the agonism of Dyn A-(1-13)NH_2 at κ opioid receptors in the AC assay with high potency (K_B = 2.6 nM). Thus we have successfully identified highly selective, potent, and stable [NMePhe^1]arodyn analogs for κ opioid receptors. [CH_3OCO-NMePhe^1]arodyn could prove to be quite useful as a pharmacological tool to compliment the non-peptide κ receptor selective antagonists to study the physiological processes mediated by κ opioid receptors and also as a lead peptide for further modification.

The N-terminal "message" sequence of arodyn is not only important for κ opioid receptor affinity but also efficacy. While arodyn is a potent and selective neutral κ opiod receptor antagonist, some analogs exhibit either inverse agonist activity or full agonist activity at κ opiod receptors.[1, 2] Structure-activity relationships (SAR) for opioid receptor affinity, selectivity, and efficacy were explored in positions 1 and 3 of arodyn (Chapter 5).

Kappa opioid receptors generally tolerate bulky aromatic amino acids at position 3 of arodyn with minimal loss in affinity. Substitution of Nal(2') in both positions 1 and 3 (compound **2**) results in a 2-fold increase in κ opioid receptor affinity. However, incorporation of a bulky aromatic amino acid at position 3 and/or 1 generally increases mu (μ) opioid receptor affinity, therefore decreasing selectivity for κ over μ opioid receptors compared with arodyn. A bulky aromatic ring in position 3 (such as Trp or Nal(2')) of arodyn appears to be important for the observed inversed agonist activity of selected arodyn analogs, while the residue in position 1 is not important for inverse agonist activity (Chapter 5). [Nal(2')^{1,3}]arodyn shows dose-dependent inverse agonist activity with high potency (EC_{50} = 9.0 nM) at κ opioid receptors.

[Tic[1],Cha[3]]arodyn and its Lys[4] analog are the only two arodyn analogs that show almost full agonist activity at κ opioid receptors.[1] The combination of the modifications of both the first and third residues are required for the agonist activity. These two compounds also show increased affinity (3.5- and 10-fold, respectively) for κ opioid receptors compared to arodyn. The efficacy might be due to the changes in residue 3 (from Phe to Cha) shifting the position of Tic[1] in the binding site such that hydrogen bonding of the N-terminal moiety with a group on the receptor changes the receptor conformation and results in receptor activation. Changing of the configuration of the first amino acid Tic could alter or eliminate a possible hydrogen bond between the N-terminal acetyl group with κ opioid receptors so that [D-Tic[1],Cha[3]]arodyn is no longer an agonist.

Substitution of the N-terminal acetyl moiety Ac-Tic[1] in [Tic[1],Cha[3]]arodyn with phenylproprionic acid (Ppa), its 2-methyl derivative 2-MePpa, and 1,2,3,4-tetrahydro-2-naphthoic acid (Tna) prevents H-bonding, and therefore could eliminate the agonist activity. These analogs generally exhibit higher affinity for κ opioid receptors and similar affinity for μ opioid receptors compared to arodyn (Chapter 5). [(S/R)-2-MePpa[1],Cha[3]]arodyn exhibits the highest affinity (K_i = 2.7 nM) and selectivity (K_i ratio (κ/μ) = 1/745) for κ opioid receptors. This mixture does not inhibit AC activity, consistent with our hypothesis, and therefore is a potential antagonist for κ opioid receptors. The two enantiomers of 2-MePpa have been resolved and incorporated into the peptides as single isomers. The pharmacological activities of these peptides will be evaluated shortly (Chapter 5).

We are specifically interested in developing conformationally constrained peptide ligands for κ opioid receptors. In an effort to reduce conformational flexibility of arodyn, various arodyn analogs cyclized by different strategies were designed (Chapters 6 and 7).

Based on the SAR and sequence of arodyn, a series of side chain-to-side chain analogs cyclized by using lactam bond were synthesized (Chapter 6). The cyclizations in the N-terminal "message" sequence involving residues 2 and 5 and longer-range cyclizations involving residues 2 and 8 of arodyn are unfavorable for κ opioid receptor binding, while cyclizations in the C-terminal "address" sequence of arodyn involving residues 5 and 8 are tolerated by κ opioid receptors. *cyclo*[Asp^5,Dap^8]arodyn (Dap = 2,3-diaminopropionic acid) retains high binding affinity (K_i = 20 nM) and exhibits about 3-fold higher selectivity for κ over μ opioid receptors than arodyn. It also exhibits no agonist activity in the adenylyl cyclase assay. This compound is the first conformationally constrained analog of arodyn with high affinity at κ opioid receptors and is a promising lead peptide that can be further modified to increase affinity and to study its interactions with κ opioid receptors. The first step towards a systematic SAR studies will be to modify the ring size. Such modifications will provide information with regard to the optimal ring size required for activity as it may alter the backbone conformation and orientation of the side chains. This can be accomplished by changing the amino acid Dap^8 to Dab (2,4-diaminobutyric acid) and Orn. These two compounds (*cyclo*[Asp^5,Dab^8]arodyn and *cyclo*[Asp^5,Orn^8]arodyn) have been synthesized and purified and are currently undergoing pharmacological assays (Chapter 6).

Cyclic arodyn analogs were also designed and synthesized using ring-closing metathesis (RCM) (Chapter 7). Cyclization of peptides through RCM offers several

advantages over conventional methods of cyclization, i.e. through amide or disulfide bond formation. The resultant carbon-carbon bond is more stable than an amide or disulfide bond.[3, 4] Furthermore, in contrast to cyclization via amide or disulfide bond formation, side chain functionalities can be retained in cyclization by RCM by appropriate choice of amino acid side chains for cyclization.

Like cyclizations involving a lactam bond, cyclizations in the N-terminal "message" sequence of arodyn involving residues 2 and 5 using RCM between two AllGly residues are unfavorable for κ opioid receptor binding. The *cis* and *trans* isomers of *cyclo*[Ala^2(-CH=CH-)Ala^5,Ile^8]arodyn show about 13- and 41-fold lower affinity for κ opioid receptors, respectively, compared to arodyn. Surprisingly, these cyclic arodyn analogs are partial or full agonists at κ opioid receptors in the adenylyl cyclase assay (38-112% intrinsic activity compared to Dyn A(1-13)-NH_2). This is very interesting since *cyclo*[Asp^2,Dap^5,Ile^8]arodyn is a neutral ligand that does not inhibit adenylyl cyclase activity. The differences probably arise from the different types of cyclizations having different effects on backbone conformation that affects the orientation of important residues.

Cyclization in the N-terminal "message" sequence of arodyn involving the residues 2 and 3 using RCM between two Tyr(All) residues is tolerated by κ opioid receptors (Chapter 7). The *trans* isomer of *cyclo*[Tyr^2(CH_2-CH=CH-CH_2)Tyr^3,Ile^8]arodyn shows only 5.5-fold lower affinity (K_i = 55 nM) compared to arodyn (K_i = 10 nM). This is the first arodyn analog cyclized through RCM that exhibits high affinity at κ opioid receptors and is an excellent lead peptide. This is particularly interesting, since the cyclic peptide did not involve any further modifications. We expect that *cyclo*^{2,3}[Tyr^2(CH_2-

CH=CH-CH_2)Tyr^3]arodyn with D-Ala in position 8 will have higher affinity based on the SAR of linear analogs. This compound will be synthesized in the near future.

We also applied RCM to the synthesis of cyclic Dyn A analogs to constrain its conformation and to synthesize agonist analogs for κ opioid receptors (Chapter 8). During this research several high affinity conformationally constrained analogs of Dyn A were synthesized. The two isomers of the N-terminal conformationally constrained Dyn A analog *cyclo*[D-Ala^2(-CH=CH-)Ala^5]Dyn A(1-11)NH_2 retain high affinity for κ, μ, and δ receptors, indicating that the conformations which are adopted by these two peptides are still compatible with interaction with all three opioid receptor types. These two peptides show very low selectivity for κ over μ receptors and only moderate selectivity for κ over δ receptors.

The two isomers of *cyclo*[Ala^5(-CH=CH-)Ala^8]Dyn A(1-11)NH_2 show intermediate affinity for κ opioid receptors; however, their selectivity over other opioid receptors is greater. The two isomers of *cyclo*[Ala^5(-CH=CH-)Ala^8]Dyn A(1-11)NH_2, with the L-configuration in position 2, show the lowest affinity for κ opioid receptors; however, these two compounds exhibit the highest selectivity for κ receptors compared to the parent peptide. In both these two cases, the configuration of the double bond has a significant influence on opioid receptor affinity and agonist potency. Similar to Dyn A, these cyclic Dyn A analogs exhibit dose-dependent agonist activity (≥ 90% compared to Dyn A-(1-13)NH_2) at κ opioid receptors with potencies corresponding to their affinities at these receptors. These analogs represent interesting lead compounds for further characterization of conformation-activity relationships for Dyn A at κ opioid receptors.

Future work involves conformational analysis of the cyclic arodyn and Dyn A analogs by nuclear magnetic resonance (NMR) and molecular modeling. These studies will provide us valuable information on possible conformational preference of these peptides for interaction with κ opioid receptors. The results from the conformational analyses of arodyn and Dyn A analogs may provide new insights into how they might bind to κ opioid receptors.

The research in this dissertation involved an extensive amount of synthetic and analytical work. Novel synthetic strategies for RCM were developed for the synthesis of cyclic arodyn analogs involving Tyr(All). (Chapter 7) By using Tyr(All) for cyclization, the important aromatic residues of arodyn could be constrained without loss of the aromatic functionalities. However, the cyclization is dependent on the linear and cyclic peptide sequences and conformations. For the cyclization to be successful, the two double bonds of the linear precursor peptide must be in close proximity. Also the product should not have significant ring strain that would require high activation energy for cyclization. Interestingly, for the successfully synthesized peptides, only the *trans* isomer was observed as confirmed by NMR. A side reaction involving the deletion of the allyl group from Tyr competes with the cyclization reactions. (Chapter 7) A mechanism for this side reaction has been proposed, in which the double bond of the allyl group migrates under the metathesis conditions to form the vinyl ether intermediate, which is then hydrolyzed under acidic cleavage conditions to form the des-OAll analog.

Additional research focused on synthesizing modified Dyn A analogs (Appendices 1 and 2) In order to synthesize the C-terminal ethylated amide Dyn A analog E-2078,[5, 6] we designed a convenient method of synthesizing C-terminal alkylated amide

peptides on the PAL-PEG-PS resin, a commonly used resin for synthesis of C-terminal amide peptides. (Appendix 1) The installation of the C-terminal alkylated amide was accomplished by a Fukuyama-type amine synthesis followed by coupling of the C-terminal amino acids.[7, 8] The alkylation reactions are very efficient with different reactive alkylating reagents when the leaving group is iodide or bromide. However, the deprotection of *o*NBS and the coupling of the first amino acid to the resulting modified resin requires extended reaction times because of increased steric hindrance. We used the non-volatile 2,2'-(ethylenedioxy)diethanethiol as the thiol to deprotect the oNBS group. The Fmoc-PAL-PEG-PS resin is compatible with the reaction conditions since the alkylation and *o*NBS deprotection are conducted under basic conditions. The synthesis of the model peptides using this method gives consistently high yields and purities. E-2078 was also successfully synthesized by this method.

In order to study the translocation of dynorphins and arodyn across the plasma membrane, we designed and synthesized analogs of these peptides with a fluorescent tag (Appendix 2). The N-terminal "message" sequences of Dyn A and arodyn are important for receptor binding. Therefore we designed analogs where 5-TAMRA was attached in the C-terminus of these peptides. These fluorescent Dyn A, Dyn B, and arodyn analogs are currently undergoing pharmacological assays. These compounds could be important pharmacological tools for the study of ligand (both agonist and antagonist)-receptor interactions as well as the penetration of peptides through biological membranes. Two of these fluorescent peptides have been used for the study of the penetration of Dyn A and Dyn B into neurons and non-neuronal cells using confocal fluorescence microscopy.[9]

This dissertation research has identified several selective, potent, and/or conformationally constrained κ opioid receptor peptides. These selective κ opioid receptor peptide agonists and antagonists can be used as pharmacological tools to complement the nonpeptide κ receptor selective ligands in studying κ opioid receptors and the physiological and pharmacological actions of agonists.

Selected peptides obtained from this dissertation should be studied in the future for their transport and metabolism. Our previous research with Prof. Susan Lunte at the Department of Pharmaceutical Chemistry and Prof. Jay McLauglin in Northeastern University suggests that the transport and/or metabolism properties of peptides can be improved by different structural modifications, including N-terminal acetylation, N-alkylation, D-amino acid substitution, and/or cyclization. Arodyn is active in the CNS when administered via intracerebroventricular (i.c.v.) injection in mice models.[10] *cyclo*[Asp^5,Dap^8]arodyn is expected to have higher metabolic stability compared to arodyn because the ring constraint can block endopeptidase cleavage in the middle of arodyn. Also both the N- and C-terminus of this peptide are blocked by acetylation and amidation, respectively.

Peptide-based ligands are excellent tools for studying ligand-receptor interactions, which can eventually aid in development of new therapeutic agents. Further, with the latest developments in the field of drug delivery, peptide-based ligands are rapidly emerging as potential therapeutic agents.

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Appendix 1. A Convenient Approach to Synthesizing C-Terminal Alkylated Amide Peptides: Synthesis of E-2078

*Note that the compound numbers used in this appendix are only applicable within this specific appendix.

A1.1 Introduction

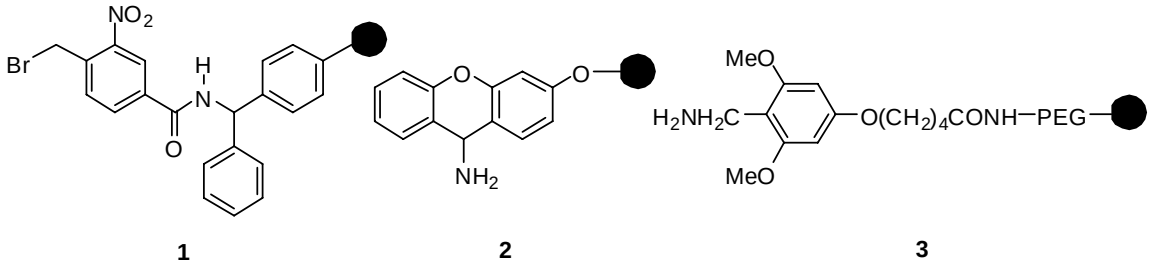
Solid-phase peptide synthesis (SPPS), first developed by Bruce R. Merrifield,[1] is a remarkable breakthrough for the synthesis of peptides and other biologically active compounds. SPPS almost always starts from the C-terminus of the peptide. Due to this fact, modifications of peptides at the C-terminus are usually more difficult than modifications at the N-terminus.

C-Terminal amide alkylation of peptides has been observed to have significant influence on their biological properties, because C-terminal alkyl amide groups such as an ethyl amide can increase stability towards peptidases.[2] C-Terminal alkylation can also increase affinity towards specific biological targets. In the case of lutenizing hormone-releasing hormone (LH-RH), several N-ethyl and N-methyl amide derivatives of LH-RH analogs are reported to be 4-5 times more active than the corresponding non-alkylated amide peptides.[3, 4] Lastly, C-terminal alkylation can increase lipophilicity and reduce hydrogen bonding potential, which could facilitate the penetration of peptides across biological membranes and/or improve pharmacokinetic properties.[5, 6] Therefore, C-terminal amide alkylation has potential pharmaceutical applications.

Given the interest in these modified peptides, a number of approaches to the synthesis of C-terminal alkylated amide peptides have been described. However, the synthesis of this type of modified peptides on solid phase is difficult. One approach is to first synthesize the corresponding peptides with a C-terminal carboxylic acid. Following cleavage from the resin the free carboxylic acid is then activated and coupled to a primary or secondary amine in solution.[7] However, this approach has some limitations as other carboxylic acid groups, i.e. those on the side chains of Asp and Glu, can also react.

Furthermore, racemization may occur at the C-terminal amino acid.[8] A similar approach is aminolysis of the resin-bound ester. For example, a 4-bromomethyl-3-nitrobenzamidobenzyl polystyrene (4-bromomethyl-Nbb) resin (**1**, Figure A1.1) developed by Nicolas and co-workers was reported for the solid phase synthesis of C-terminal alkylated amide peptides.[9] However, this approach may not be compatible with Fmoc (9-fluorenylmethoxycarbonyl)/*t*Bu (*tert*-butyl) chemistry since the secondary amine piperidine, which is used for cleavage of Fmoc group during each coupling, can also aminolyze the resin-bound peptide ester.[9]

Figure A1.1: Structures of resins **1-3**



1 **2** **3**

Another general approach involves the use of a modified resin linker for suitable attachment of an alkyl amine to the resin before peptide coupling starts. For example, reductive amination of the 9-amino-xanthen-3-yloxymethylpoly(styrene) resin (**2**, Figure A1.1) followed by standard peptide synthesis affords C-terminal alkylated amides.[10] However, in the same article, it was reported that the PAL-PEG-PS (PAL = peptide amide linker; PEG = polyethylene glycol; PS = polystyrene) resin (**3**, Figure A1.1) is not applicable for the synthesis of C-terminal alkylated amide peptides, probably due to steric hindrance. Also, reductive alkylation on solid phase with an excess of aldehyde usually

results in dialkylation,[11, 12] which will decrease the yield of the desired monoalkylated products.

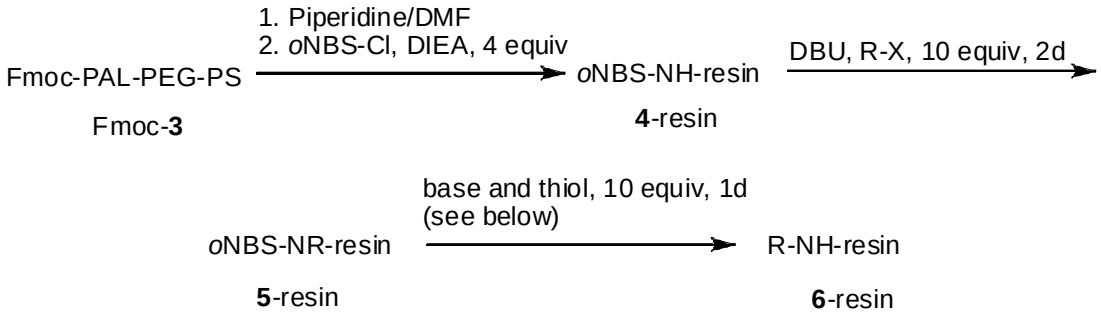
Here we report a convenient way of synthesizing C-terminal alkylated amide peptides. We applied a well-known N-alkylation reaction, the Fukuyama-type amine synthesis,[13, 14] to the synthesis of C-terminal alkylated amide peptides. This type of reaction has been applied to the synthesis of N-methylated amino acid-containing peptides by both solid and solution phase methods.[15, 16] However, to our knowledge this type of reaction has not been reported for the synthesis of C-terminal alkylated amide peptides. In this strategy, monoalkylation at the C-terminus was successfully performed on the PAL-PEG-PS resin (**3**), a commonly used resin for the synthesis of C-terminal amide peptides.

A1.2 Results and discussion

A1.2.1 Fmoc-deprotection and *o*NBS coupling

The synthesis of the C-terminal alkylated amide peptides used the Fmoc-PAL-PEG-PS resin (0.19 mmol/g, Scheme A1.1). The Fmoc group was first deprotected using piperidine in DMF (1/4, v/v) to form the free amine, which was then coupled with 4 equiv of *o*NBS-Cl (*ortho*-nitrobenzenesulfonic chloride) and DIEA (*N*,*N*-diisopropylethylamine) in DCM (dichloromethane). The completion of this reaction was monitored by the ninhydrin test.[17] Cleavage of an aliquot of the resin afforded pure *o*NBS-NH_2 (**4**) as confirmed by HPLC (5-50% MeCN over 45 min at 1 mL/min, see below, t_R = 3.8 min).

Scheme A1.1: Synthesis of RNH-PAL-PEG-PS resin



A1.2.2 Alkylation

The newly formed sulfonamide (**4**-resin) could be deprotonated by DBU (1,8-diazabicyclo[5.4.0]undec-7-ene).[16] The resulting anion was then reacted with several different alkyl halides (Table A1.1). The yields of these reactions were monitored by HPLC analysis of aliquots using both 214 and 280 nm wavelengths for appearance of *o*NBS-NHR (**5**) and disappearance of *o*NBS-NH_2 (**4**, Scheme A1.1). The results are shown in Table A1.1. The conversion was quantitative for most of the alkylating reagents (methyl iodide, ethyl iodide, allyl bromide, propargyl bromide, cyclopropylmethyl (CPM) bromide, and benzyl bromide) after reaction for 2 days (Table A1.1, entries A-F) except for phenylethyl chloride and phenylethyl bromide (Table A1.1, entries G-K). Even with potassium iodide (KI) as a catalyst and/or heating the reaction (80 ^{o}C, Table A1.1, entries H and I), there was no apparent conversion when the alkylating reagent was the relatively unreactive phenylethyl chloride. With phenylethyl bromide, which is more reactive than phenylethyl chloride, there was only 20% conversion after reaction for 2 days at room temperature (Table A1.1, entry J). Increasing the reaction temperature to 80 ^{o}C did not increase the yield (21%, Table A1.1, entry K) under the same reaction conditions.

Table A1.1: Yield of alkylation reactions with different alkyl halides

Entry	R-X	Yield (%)/t_R (min) of *o*NBS-NH-R[a]	Entry	R-X	Yield (%)/t_R (min) of *o*NBS-NH-R[a]
A	Me-I	>98/7.9	G	$PhCH_2CH_2Cl$	<5
B	Et-I	>98/12.7	H	$PhCH_2CH_2Cl$/KI	<5
C	All-Br	>98/14.9	I	$PhCH_2CH_2Cl$/KI[b]	<5
D	Propargyl-Br	>98/12.9	J	$PhCH_2CH_2Br$	20/31.4
E	CPM-Br	>98/20.1	K	$PhCH_2CH_2Br$[b]	21/31.6
F	Bn-Br	>98/27.0			

[a]The yield was determined by comparing the relative area under the peaks of *o*NBS-NH-R (**5**) in HPLC chromatograms to non-alkylated *o*NBS-NH_2 (**4**). [b]At 80 °C.

A1.2.3 *o*NBS deprotection

The *o*NBS deprotection of *o*NBS-NR-resin was relatively slow. The mechanism of this reaction is well documented in the literature.[13] We examined various deprotection conditions for *o*NBS-N(Et)-resin using different bases and thiols (Table A1.2). The completion of this reaction was monitored by the disappearance of the *o*NBS-NH-R by HPLC. 4-Dimethylaminopyridine (DMAP), a commonly used base in organic synthesis, could only partially cleave the *o*NBS group (Table A1.2, entry A). DBU was required as the base to deprotonate the thiol group, thereby increasing its nucleophilicity. This was probably due to the fact that DMAP is not as basic as DBU and therefore could not deprotonate the thiol group as efficiently. The thiols investigated (2-mercaptoethanol ($HSCH_2CH_2OH$), thiophenol (PhSH), and 2,2'-(ethylenedioxy)diethanethiol ($(HSCH_2CH_2OCH_2)_2$)) all showed similar results for deprotection of the N-ethylamide under these reaction conditions (Table A1.2, entries B-D). Therefore, the non-volatile 2,2'-(ethylenedioxy)diethanethiol and DBU were used to deprotect the *o*NBS from the

other alkylated resins. To the best of our knowledge there has been no previous report of using 2,2'-(ethylenedioxy)diethanethiol to deprotect the *o*NBS group.

Table A1.2: Exploration of the *o*NBS cleavage conditions from the *o*NBS-N(Et)-resin (base/thiol/DMF (*N*,*N*-dimethylformamide)=1/1/2, v/v/v, 1 d, rt)

Entry	Base	Thiol	Yield (%)
A	DMAP	$HSCH_2CH_2OH$	24
B	DBU	$HSCH_2CH_2OH$	>99
C	DBU	PhSH	>99
D	DBU	$(HSCH_2CH_2OCH_2)_2$	>99

While most of the resins with different alkyl groups showed complete cleavage using DBU and 2,2'-(ethylenedioxy)diethanethiol as the base and thiol, respectively (Table A1.3, entries A-E), the deprotection of the *o*NBS-N(CPM)-resin turned out to be difficult (Table A1.3, entry F). Only a trace amount of the *o*NBS was removed under standard conditions. Increasing the temperature to 80 ^oC only increased the cleavage to around 30% (entry G). This was probably due to steric hindrance by the CPM group. In contrast, the alkylation reaction, which probably proceeds through an S_N1 reaction due to stabilization of the CPM carbocation, was very efficient. Thiophenol was found to be a better reagent to cleave the *o*NBS group from this N-alkylated resin (Table A1.3, entry H). Any remaining trace amount of unreacted *o*NBS group would act as an end-capping group and would not interfere with later peptide synthesis.

Table A1.3: Cleavage of *o*NBS group from *o*NBS-N(R)-resin (DBU/2,2'-(ethylenedioxy)diethanethiol/DMF=1/1/2 at rt, 1d)

Entry	R	Yield (%)	Entry	R	Yield (%)
A	Me	Quantitative	E	Bz	Quantitative
B	Et	Quantitative	F	CPM	Trace
C	Allyl	Quantitative	G	CPM[a]	30%
D	Propargyl	Quantitative	H	CPM[b]	Quantitative

[a]At 80 ^{o}C; [b]Thiophenol used instead of 2,2'-(ethylenedioxy)diethanethiol for 1 day.

A1.2.4 Peptide synthesis, analysis, and purification

We then continued to synthesize C-terminal alkylated amide derivatives of Leu-enkephalin, Tyr-Gly-Gly-Phe-Leu-NH-R, as model peptides. The non-alkylated Tyr-Gly-Gly-Phe-Leu-NH_2 was also synthesized for comparison. Leu-enkephalin analogs were chosen because Leu-enkephalin is a relatively simple peptide and therefore a good model to evaluate this method of synthesizing C-terminal alkylated amide peptides. Furthermore, the C-terminal residue Leu is of intermediate steric bulk, and therefore can be used to evaluate the coupling efficiency to a secondary amine. Coupling of Fmoc-Leu-OH (using benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBt), and DIEA in DMF (4/4/4/10) as coupling reagents) to the secondary amino groups was relatively slow and required double couplings with extended reaction times (overnight). Any unreacted free amino groups were capped by using an excess of acetic anhydride and DIEA in DMF. Loadings of the first amino acid were determined by Fmoc quantitation (Table A1.4).[18, 19] Coupling yields of the first amino acid Leu to all of these secondary amino groups were modest (87-95%, Table A1.4). Following the successful installation of the first amino acid, the synthesis of these C-terminal alkylated amide peptides was continued using the standard SPPS method described in Chapter 3.

Table A1.4: Loading yields for the first amino acid coupling to the resins

Entry	Resin	Fmoc-loading (mmol/g)[a]	Loading yield
A	Fmoc-Leu-N(Me)-resin	0.171 ± 0.001	92%
B	Fmoc-Leu-N(Et)-resin	0.169 ± 0.004	91%
C	Fmoc-Leu-N(All)-resin	0.171 ± 0.001	92%
D	Fmoc-Leu-N(Propargyl)-resin	0.176 ± 0.003	95%
E	Fmoc-Leu-N(CPM)-resin	0.175 ± 0.001	95%
F	Fmoc-Leu-N(Bz)-resin	0.160 ± 0.001	87%

[a]The numbers are expressed as mean ± SEM of three samples.

These peptides were then cleaved with 95% TFA (trifluoroacetic acid) with 5% water for 2 hours. Electrospray ionization mass spectrometry (ESI-MS) analysis showed the desired molecular weights for all of the peptides. HPLC analysis showed that most of these crude peptides have a purity of greater than 90% (Table A1.5). There was no trace of the non-alkylated control peptide (Tyr-Gly-Gly-Phe-Leu-NH_2, standard HPLC method, see experimental section, t_R = 14.6 min) either in the HPLC or MS spectrum.

Table A1.5: HPLC and MS analysis of crude C-terminal alkylated amide peptides

Entry	Peptide sequence	[a]HPLC	ESI-MS (*m/z*, $[M+H^+]^+$)	
		t_R (min)/% Purity	Calculated	Observed
A	Y-G-G-F-L-NH_2	14.6/98.4	555.3	555.3
B	Y-G-G-F-L-NH-Me	14.8/90.4	569.3	569.3
C	Y-G-G-F-L-NH-Et	16.8/97.0	583.3	583.3
D	Y-G-G-F-L-NH-All	17.9/88.0	595.3	595.3
E	Y-G-G-F-L-NH-propargyl	17.6/98.9	593.3	593.3
F	Y-G-G-F-L-NH-CPM	20.0/90.7	609.3	609.3
G	Y-G-G-F-L-NH-Bz	23.5/93.8	645.3	645.3

[a]HPLC conditions: 5-50% solvent B over 45 min (solvent A = H_2O; solvent B = MeCN, both containing 0.1% TFA) at 1 mL/min, monitored at 214 nm.

The crude peptides were purified by preparative reversed phase HPLC using MeCN/water system (see below). The final purity of all of the peptides by both methods was greater than 98% (Table A1.6).

Table A1.6: Analytical data for purified peptides

Entry	Peptide sequence	[a]HPLC t_R (min)/% Purity	[b]HPLC t_R (min)/% Purity
A	Y-G-G-F-L-NH_2	14.9/100	24.4/98.5
B	Y-G-G-F-L-NH-Me	15.0/100	26.4/98.1
C	Y-G-G-F-L-NH-Et	17.0/100	29.6/100
D	Y-G-G-F-L-NH-All	18.5/100	31.4/100
E	Y-G-G-F-L-NH-propargyl	17.7/100	29.3/100
F	Y-G-G-F-L-NH-CPM	20.7/100	34.2/100
G	Y-G-G-F-L-NH-Bz	24.8/100	40.5/100

[a]HPLC conditions: 5-50% solvent B over 45 min (Solvent A = H_2O; Solvent B = MeCN, both containing 0.1% TFA), monitored at 214 nm. [b]HPLC conditions: 5-50% Solvent B over 45 min (Solvent A = H_2O; Solvent B = MeOH, both containing 0.1% TFA), monitored at 230 nm.

A1.2.5 Synthesis of E-2078

E-2078 (([NMe-Tyr[1],NMe-Arg[7],D-Leu-NHEt[8]]Dyn A(1-8)) is a stabilized Dyn A analog that shows comparable affinity but lower selectivity for κ receptor affinity compared to Dyn A.[20, 21] E-2078 has greatly increased stability towards peptidases because of several modifications.[20, 22] It produces analgesia following both intravenous (i.v.) and subcutaneous (s.c.) administration.[23, 24] This is the only Dyn A analog that has been through both extensive pre-clinical and clinical evaluations. Therefore we then applied the method described above to the synthesis of E-2078. The synthesis started form the Et-NH-resin (Scheme A1.2). Following the successful installment of the C-terminal ethyl amide moiety, the synthesis of E-2078 was continued until the whole peptide was assembled. The N-methyl groups on Tyr[1] and Arg[7] were installed using a similar method as the C-terminal ethyl group (Scheme A1.2). The three N-alkylation and *o*NBS deprotection reactions were relatively slow. The couplings of Fmoc-D-Leu-OH (residue 8) and Fmoc-Arg(Pbf)-OH (residue 6) to the two secondary amines were also much more difficult than regular amino acid coupling reactions. The crude peptide showed a purity of 53% by HPLC (t_R = 19.0 min, Figure A1.2).

Scheme A1.2: Synthesis of E-2078

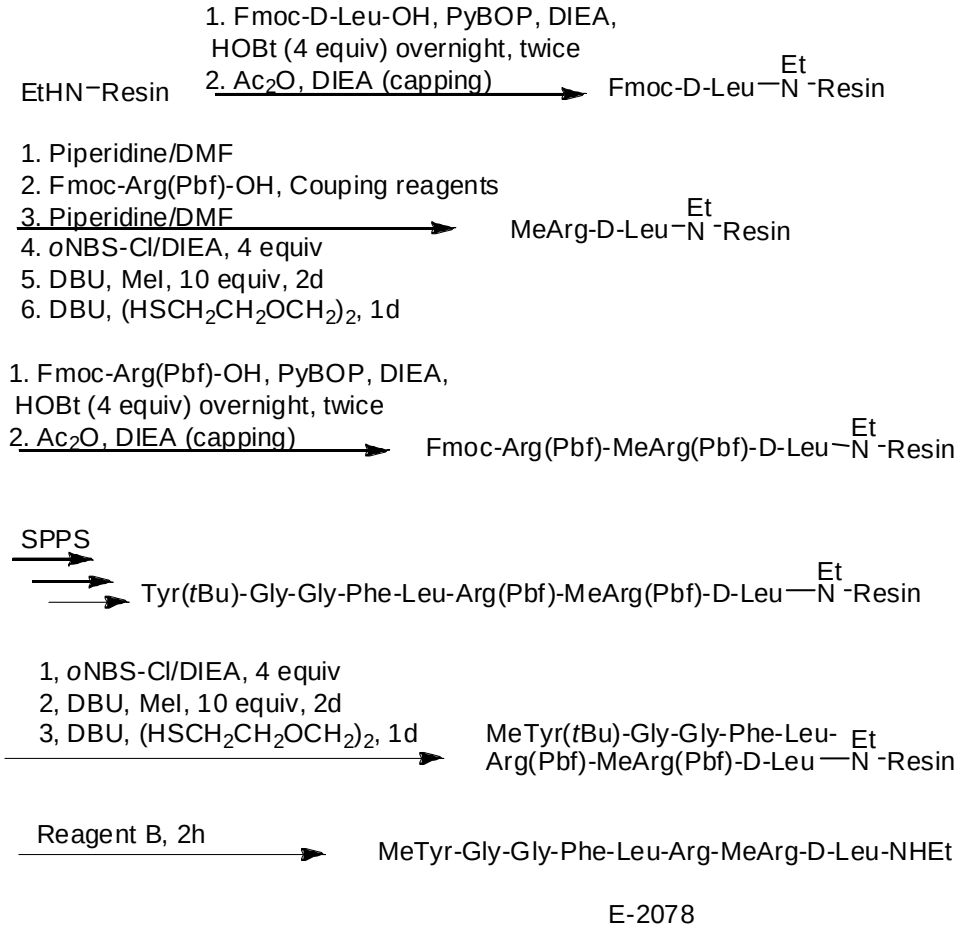
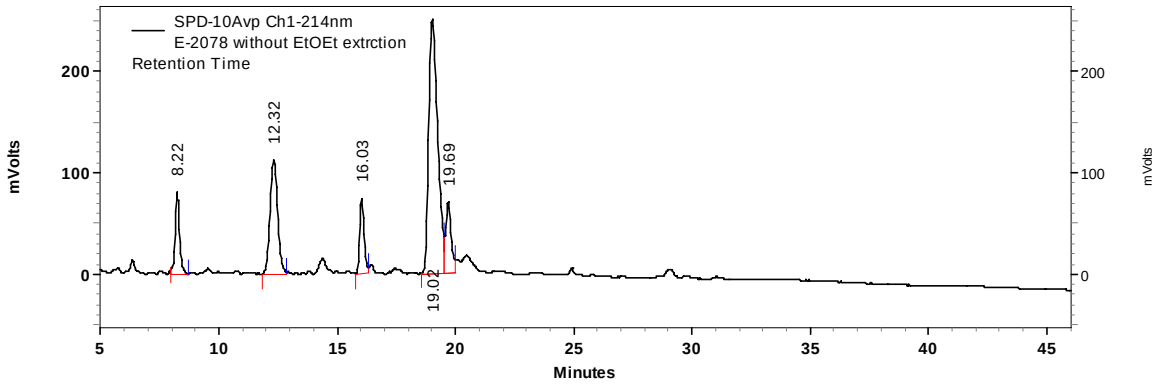


Figure A1.2: HPLC spectrum of crude E-2078



[a]HPLC conditions: 5-50% solvent B over 45 min (Solvent A = H_2O; Solvent B = MeCN, both containing 0.1% TFA), monitored at 214 nm.

Currently, there is one commercially available resin, namely the "safety catch resin", used for the synthesis of these modified peptides.[25-27] The method described here has some advantages. It is especially useful for the synthesis of combinatorial peptide libraries with modifications at the C-terminus as the C-terminal alkyl group is installed in the first stage, which is the rate-limiting step. Following the coupling of the first amino acid, the remainder of the peptides could be assembled on automatic synthesizers. Another advantage is that the PAL-PEG-PS resin used has improved physicochemical properties because of the incorporation of the PEG group compared to the safety catch resin, which is a polystyrene resin.

A1.3 Conclusions

In conclusion, we applied a well-known type of N-alkylation reaction to the efficient synthesis of C-terminal alkylated amide peptides. The alkylation reaction was very efficient with different reactive alkylating reagents when the leaving group was iodide or bromide. However, the deprotection of *o*NBS and the coupling of the first amino acid to the resins required extended reaction times because of increased steric hindrance. We were the first to use the non-volatile 2,2'-(ethylenedioxy)diethanethiol as the thiol to deprotect the oNBS group. The PAL-PEG-PS resin was compatible with the reaction conditions since the alkylation and *o*NBS deprotection were conducted under basic conditions. The synthesis of the model peptides using this method gave consistently high yields and purities. E-2078 was also successfully synthesized by this method. Since the alkyl groups are introduced in the first step, this method can be widely applied in combinatorial synthesis of C-terminal alkylated amide peptides.

A1.4 Experimental section

A1.4.1 Materials

*o*NBS-Cl, DBU, DMAP, ethyl iodide, cyclopropylmethyl bromide, phenylethyl bromide, potassium iodide, 2-mercaptoethanol, and 2,2'-(ethylenedioxy)diethanethiol were purchased from Sigma-Aldrich (Milwaukee, WI). Methyl iodide was purchased from Fisher Scientific (Hampton, NH). Allyl bromide, benzyl bromide, propagyl bromide, phenylethyl chloride, thiophenol and 4-dimethylaminopyridine (DMAP) were purchased from Acros Chemical Co. (Pittsburgh, PA). The sources of other materials are listed in Chapter 3.

A1.4.2 Synthesis of C-terminal alkyl group

The Fmoc group of the Fmoc-PAL-PEG-PS resin (Fmoc-**3**, 0.19 mmol/g) was first deprotected using piperidine in DMF (1/4, v/v, 2 X 10 min) to form the free amine, which was then coupled with 4 equiv of *o*NBS-Cl and DIEA in DCM for 30 min to afford the *o*NBS-NH-resin (**4**-resin, Scheme A1.1). The completion of this reaction was monitored by the ninhydrin test.[17] Cleavage of an aliquot of the resin afforded pure *o*NBS-NH_2 (**4**, t_R = 3.8 min). The *o*NBS-NH-resin was then treated with an alkyl halide and DBU (10 equiv each) in DMF for 2 days to afford the *o*NBS-NR-resins (**5**-resin). The yields of these reactions were monitored by HPLC analysis (5-50% MeCN containing 0.1% TFA over 45 min at 1 mL/min) of aliquots using 214 nm as the wavelength to monitor the appearance of *o*NBS-NHR and the disappearance of *o*NBS-NH_2 following cleavage from the resin using TFA. For phenylethyl chloride, KI (0.1 equiv) was also

added as a catalyst; the reaction was run at room temperature or at 80 ^{o}C to increase the conversion rate. The *o*NBS-NR-resins (**5**-resins) with quantitative alkylation were then treated with different bases and thiols in DMF (1/1/2) at room temperature or 80 ^{o}C for 1 day to afford the RNH-resins (**6**-resins). A small quantity of resin (around 5 mg) was cleaved using TFA (1 mL) for 3 hours. Following filtration and washing of the resin with TFA (1 mL), the TFA was evaporated using air. The residue was then dissolved in 100 μL acetonitrile/water (50/50) and 5 uL of the mixture was analyzed by HPLC (5-50% MeCN containing 0.1% TFA over 45 min at 1 mL/min). The difference in the absorbance of *o*NBS-NHR before and after *o*NBS deprotection was used to estimate the conversion rate.

A1.4.3 Synthesis of the model peptides

Fmoc-Leu-OH (4 equiv) was double-coupled to the **6**-resins with PyBOP, HOBt, and DIEA in DMF (4/4/10) for 12 h. The reaction was monitored using the chloranil test.[28] Any unreacted free amino groups were capped using an excess of acetic anhydride and DIEA (20 equiv each) in DMF. Loadings of the first amino acid were determined by Fmoc quantitation (see below).[18, 19] Following the successful installation of the first amino acid, the synthesis of these C-terminal alkylated amide peptides was continued using the standard SPPS method as described in Chapter 3.

A1.4.4 Fmoc quantitation[18, 19]

About 5 mg of resin (three samples for each resin) was weighed into a 10 mL volumetric flask. To the flask was added DCM (0.4 mL) and piperidine (0.4 mL), and the

reaction was allowed to proceed for 30 min. Then methanol (1.6 mL) was added, followed by DCM to a total of 10 mL. The UV absorbance was measured at 301 nm three times compared to a blank solution containing of piperidine (0.4 mL), MeOH (1.6 mL), and DCM to make 10 mL. The Fmoc loading was calculated based on the following equation 1:

$$\text{Loading (mmol/g)} = 1000*C*V/m = 1000*(Abs/(\varepsilon*l))*V/m \quad (1)$$

where Abs is the average absorbance of the three readings, V = 10 mL, ε is the extinction coefficient (7800 $M^{-1}cm^{-1}$), l is the length of UV cell (1), and m is the amount of resin.

The theoretical substitution of a peptidyl resin can be calculated from the substitution of the base resin using the following equation 2:

$$\text{Theoretical loading (mmol/g)} = A * 1000/[1000 + (A * (M - X)] \quad (2)$$

where A is the substitution of starting resin (mmol/g); M is the molecular weight of Fmoc-Leu-NHR; and X is 239 as the resin is initially protected with Fmoc.

A1.4.5 Synthesis of E-2078

E-2078 was synthesized in a similar manner (Scheme A1.2). The N-methyl groups of Tyr^1 and Arg^7 were installed using the same method as the C-terminal ethyl group (Scheme A1.2). Fmoc-D-Leu-OH (residue 8) and Fmoc-Arg(Pbf)-OH (residue 6, 4 equiv) were double coupled with PyBOP, HOBt, and DIEA in DMF (4/4/10) for 12 h each.

A1.4.6 Cleavage and analysis of peptides

The model peptides (Y-G-G-F-L-NHR) were cleaved from the resins using 95% TFA and 5% water for 2 h. The TFA was then evaporated using air. E-2078 was cleaved using reagent B[29] under standard conditions as described in Chapter 3. All of the crude peptides were analyzed by HPLC. A linear gradient of 5-50% solvent B (solvent A aqueous 0.1% TFA, and solvent B MeCN containing 0.1% TFA) over 45 min, at a flow rate of 1 mL/min, was used for analysis. The UV wavelength 214 nm was used to quantify the yields of the desired products. The molecular weights of these peptides were determined by ESI-MS.

A1.4.7 Purification of peptides

The crude peptides were purified by preparative reversed phase HPLC. For purification a linear gradient of 15-50% aqueous MeCN containing 0.1% TFA over 35 min, at a flow rate of 20 mL/min, was used. The purity of the final peptides was verified by two analytical HPLC systems. For analytical HPLC using the MeCN system, a linear gradient of 5-50% solvent B (solvent A, aqueous 0.1% TFA and solvent B, MeCN containing 0.1% TFA) over 45 minutes, at a flow rate of 1 mL/min, was used for analysis. For the MeOH system, a linear gradient of 5-50% solvent B (solvent A, aqueous 0.1% TFA and solvent B, MeOH containing 0.1% TFA) over 45 minutes, at a flow rate of 1 mL/min, was used.

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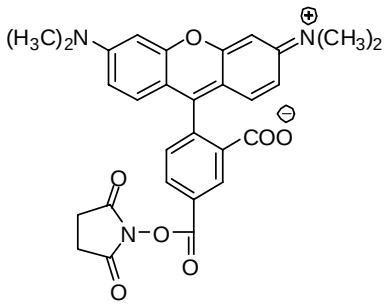
Appendix 2. Design and Synthesis of Fluorescent Dyn A, Dyn B, and Arodyn Analogs

*Note that the compound numbers used in this appendix are only applicable within this specific appendix.

A2.1 Introduction

Dynorphin (Dyn) A, Dyn B and arodyn are similar to cell penetrating peptides in possessing a high number of basic amino acid residues. In order to study the translocation of these peptides across the plasma membrane, we designed and synthesized fluorescent analogs of these peptides with a fluorescent tag attached to either the N- or C-terminus. The structure of 5-carboxytetramethylrhodamine succinimidyl ester (5-TAMRA-SE), as shown in Figure A2.1, is highly conjugated and is widely used as an amine-reactive probe.[1-3] Here we use 5-TAMRA-SE to label Dyn A, Dyn B, and arodyn for the study of the absorption of these peptides by cells.

Figure A2.1: Structure of 5-TAMRA-SE



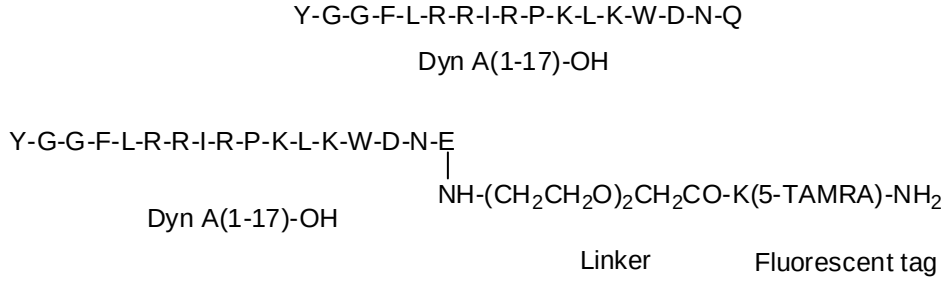
From a synthetic point of view, attachment of the fluorescent tag onto the N-terminus of Dyn A (**1**, Table A2.1) is easier to accomplish than to the C-terminus. However, the N-terminal free amine of Tyr^1 of Dyn A is critical for receptor binding, and therefore the modification of the amine with a bulky fluorescent group can affect the pharmacological activity.[4] Plus, the elimination of the positive charge can change the physiochemical properties of the peptide; this is particularly a concern for examining cellular uptake of the endogenous opioid peptides. An alternative position of adding the

fluorescent tag is residue 2 (**2**, Table A2.1). Gly^2 can be replaced with Lys, and the free amine of the Lys side chain can be attached to the fluorescent tag. Although this modification has some advantages over N-terminal attachment, the bulky fluorescent group in the important N-terminal "message" sequence may still affect the pharmacological activity. Furthermore, substitution of an L-amino acid in the second position of Dyn A can decrease kappa (κ) opioid receptor affinity.[5] Therefore we also designed an analog where 5-TAMRA was attached to the C-terminus of Dyn A (**3**, Table A2.1). A poly(ethylene glycol) (PEG)-like linker (-$NH(CH_2CH_2O)_2CH_2CO$-) was used to attach Lys(5-TAMRA) to Dyn A to minimize the influence of the bulky fluorescent tag on the interactions between Dyn A and κ opioid receptors. All of the important functionalities of Dyn A could be preserved in this way (Figure A2.2).

Table A2.1: Structures of Dyn A, Dyn B, arodyn and fluorescent peptides

Compound	Structure
Dyn A	Y-G-G-F-L-R-R-I-R-P-K-L-K-W-D-N-Q
1, 5-TAMRA-Dyn A	5-TAMRA-Y-G-G-F-L-R-R-I-R-P-K-L-K-W-D-N-Q
2, [Lys(5-TAMRA)^2]Dyn A	Y-K(5-TAMRA)-G-F-L-R-R-I-R-P-K-L-K-W-D-N-Q
3, [Glu(Linker-Lys(5-TAMRA)-NH_2)^{17}]Dyn A	Y-G-G-F-L-R-R-I-R-P-K-L-K-W-D-N-E(NH-$(CH_2CH_2O)_2CH_2CO$-Lys(5-TAMRA)-NH_2)
Dyn B	Y-G-G-F-L-R-R-N-F-K-V-V-T
4, 5-TAMRA-Dyn B	5-TAMRA-Y-G-G-F-L-R-R-N-F-K-V-V-T
5, [Glu(Linker-Lys(5-TAMRA)-NH_2)^{13}]Dyn B	Y-G-G-F-L-R-R-N-F-K-V-V-E(NH-$(CH_2CH_2O)_2CH_2CO$-Lys(5-TAMRA)-NH_2)
Arodyn	Ac-F-F-F-R-L-R-R-a-R-P-K-NH_2
6, Arodyn-Linker-Lys(5-TAMRA)-NH_2	Ac-F-F-F-R-L-R-R-a-R-P-K-$NH(CH_2CH_2O)_2CH_2CO$-Lys(5-TAMRA)-NH_2

Figure A2.2: Structural comparison of Dyn A and the analog with the fluorescent attached in the C-terminus (**3**)



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Dyn B is another important endogenous opioid peptide for κ opioid receptors (Table A2.1). Structurally it is very similar to Dyn A; the N-terminal sequences are the same in these two peptides and they both have a high number of basic residues in the C-terminal sequence. 5-TAMRA was attached to both the N- and the C-terminus (**4** and **5**, Table A2.1) of Dyn B in similar ways as described for Dyn A.

Arodyn (Ac[Phe^{1,2,3},Arg^{4},D-Ala^{8}]Dyn A(1-11)NH_2) is an acetylated Dyn A analog identified in our laboratory that is a potent and highly selective κ opioid receptor antagonist.[6] Arodyn shows significant antagonist activity in mice for up to 3 days following intracerebroventricular (i.c.v.) administration;[7] however, it shows rapid disappearance in brain homogenate (i.e. less than 2 min).[8] These contradictory results may be explained upon further study of the transport and metabolism of arodyn *in vivo*. Previous results suggest that the N-terminal "message" sequence of arodyn is important for κ opioid receptor affinity.[9] Therefore we synthesized the fluorescent arodyn analog (**6**, Table A2.1) where the fluorescent tag (5-TAMRA) was attached to the C-terminus separated by the same PEG-like linker (-$NH(CH_2CH_2O)_2CH_2CO$-) used for the synthesis of fluorescent Dyn A analog **3** and Dyn B analog **5**.

A2.2 Results, discussion, and conclusions

The endogenous peptides Dyn A and Dyn B have carboxylic acids at their C-termini. Therefore we wanted to retain the acidic functionality to maintain the physicochemical properties of these peptides. Dyn A has Gln in position 17, and therefore this side chain can be used as an attachment point to a PEG-like linker which is then attached to a fluorescent tag (Figure A2.2). This way the side chain functionality (Gln) as well as the C-terminal carboxylic acid group can be maintained. This can be accomplished by coupling of Fmoc-Glu-O*t*Bu instead of Fmoc-Gln(Trt)-OH to the resin at residue 17. We used a similar strategy to maintain the acidic functionality of Dyn B by substituting the C terminal Thr with Glu and using the side chain carboxylic acid to attach the linker and the fluorescent tag.

These fluorescent peptides (**1**-**6**) were synthesized and purified to >96% purity as determined by high-performance liquid chromatography (HPLC) in two solvent systems. The molecular weights were confirmed by electrospray ionization mass spectrometry (ESI-MS). The analytical data for these peptides is shown in Table A2.2.

These fluorescent Dyn A, Dyn B, and arodyn analogs are currently undergoing pharmacological evaluation. Peptides **1** and **4** have been used for the study of the penetration of Dyn A and Dyn B into neurons and non-neuronal cells using confocal fluorescence microscopy.[10] These compounds could be important pharmacological tools for the study of ligand (both agonist and antagonist)-receptor interactions as well as the penetration of peptides through biological membranes.

Table A2.2: Analytical data for the purified fluorescent peptides

Peptides	HPLC (t_R (min)/% Purity)		ESI-MS (*m*/z)	
	System 1[a]	System 2[b]	Calculated	Observed
1	26.96/100	33.48/100	$[M+4H]^{4+}$=640.9 $[M+3H]^{3+}$=854.2 $[M+2H]^{2+}$=1280.8	$[M+4H]^{4+}$=640.9 $[M+3H]^{3+}$=854.2 $[M+2H]^{2+}$=1280.8
2	25.06/100	32.22/100	$[M+4H]^{4+}$=658.4 $[M+3H]^{4+}$=877.5 $[M+5H]^{4+}$=526.9	$[M+4H]^{4+}$=658.5 $[M+3H]^{4+}$=877.7 $[M+5H]^{4+}$=527.0
3	25.28/96.5	30.90/97	$[M+3H]^{3+}$=944.8 $[M+4H]^{4+}$=708.9	$[M+3H]^{3+}$=944.8 $[M+4H]^{4+}$=708.9
4	25.32/100	31.30/100	$[M+3H]^{3+}$=662.0 $[M+4H]^{4+}$=496.8 $[M+2H]^{2+}$=992.5	$[M+3H]^{3+}$=662.2 $[M+4H]^{4+}$=496.8 $[M+2H]^{2+}$=992.7
5	24.41/100	29.90/99.1	$[M+3H]^{3+}$=761.7 $[M+2H]^{2+}$=1142.6 $[M+4H]^{4+}$=571.8	$[M+3H]^{3+}$=761.7 $[M+2H]^{2+}$=1142.6 $[M+4H]^{4+}$=571.8
6	27.15/98	30.81/100	$[M+5H]^{5+}$=444.9 $[M+TFA+4H]^{4+}$=584.3 $[M+TFA+3H]^{3+}$=778.7	$[M+5H]^{5+}$=444.8 $[M+TFA+4H]^{4+}$=584.3 $[M+TFA+3H]^{3+}$=778.7

[a]System 1: 5-50% Solvent B over 45 min at 1 mL/min (Solvent A= H_2O; Solvent B= MeCN, both containing 0.1% TFA); [b]System 2: 5-77% Solvent B over 45 min at 1 mL/min (Solvent A= H_2O; Solvent B= MeOH, both containing 0.1% TFA)

A2.3 Experimental section

A2.3.1 Materials

Fmoc-Gln(Trt)-Wang resin (Trt = trityl) was purchased from Bachem (King of Prussia, PA). Fmoc-Thr(*t*Bu)-Wang resin was purchased from Calbiochem-Novobiochem (San Diego, CA). Fmoc-Glu-O*t*Bu was purchased from Bachem. Fmoc-Lys(Alloc)-OH (Alloc = allyloxycarbonyl) was purchased from Applied Biosystems (Foster City, CA). 5-TAMRA-SE was purchased from AnaSpec (San Jose, CA). Tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) was purchased from Acros Chemical Co. (Pittsburg, PA). The linker Fmoc-$NH(CH_2CH_2O)_2CH_2COOH$ (**7**) was synthesized by Dr. Xin Wang in our laboratory following the method developed previously in our laboratory.[11] The sources of other materials are listed in Chapter 3.

A2.3.2 Synthesis of 1, 2 and 4

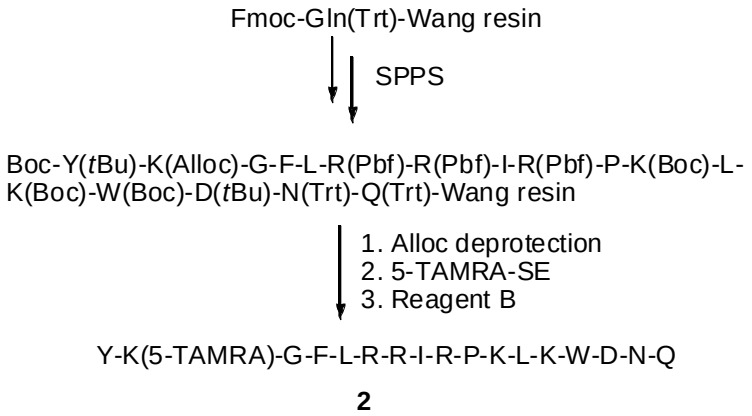
The two fluorescent Dyn A analogs **1** and **2** were synthesized by solid-phase peptide synthesis (SPPS) using Fmoc-protected amino acids on the Fmoc-Gln(Trt)-Wang resin (200 mg, 0.43 mmol/g) in a similar way as described in Chapter 3. The Wang resin is more hydrophobic than the PAL-PEG-PS resin, and thus instead of using piperidine/DMF (1/4) as the deblocking reagent, piperidine/DMF/toluene (30/35/35) was used.[12] After residues 3-16 were attached, a resin aliquot was cleaved for analysis, and the loading was determined by Fmoc-quantitation (actual loading = 0.18 mmol/g, theoretical loading = 0.18 mmol/g; the procedure for Fmoc quantitation[13, 14] was described in Appendix 1 (Section A1.4.3) of this dissertation). The resin was then separated into two portions. For the synthesis of 5-TAMRA-Dyn A (**1**), Fmoc-Gly-OH and Fmoc-Tyr(*t*Bu)-OH were coupled sequentially to the resin (31 mg, 0.0056 mmol). The fluorescent group 5-TAMRA was then attached to the free amino group of the Tyr residue by reacting with 5-TAMRA-SE (2.5 mg, 0.005 mmol, 0.9 equiv) in DMF (1 mL) for 48 h. The resin was then washed successively with DCM/DMF (1/1, 5 X 30 min), and finally with methanol to shrink the resin.

For the synthesis of [Lys(5-TAMRA)^2]Dyn A (**2**), Fmoc-Lys(Alloc)-OH and Boc-Tyr(*t*Bu)-OH (2-fold excess each) were coupled sequentailly to the resin (200 mg, 0.0368 mmol) using benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBt) and *N*,*N*-diisopropylethylamine (DIEA) as the coupling reagents (1/1/2 compared to the amino acids) (Scheme A2.1). The Alloc group attached to Lys was deprotected using $Pd(Ph_3P)_4$ (4.3 mg, 0.0037 mmol, 0.1 equiv) and phenylsilane ($PhSiH_3$, 95 mg, 0.88 mmol, 24 equiv) in DCM (5 mL, 2 X 30 min).[15] The

following solvents were used to wash the resin after the reaction: 5 X 2 min DCM, 4 X 2 min DMF, 3 X 2 min DCM, 3 X 2 min DMF, 3 X 2min 0.5% DIEA in DMF, 3 X 15 min 0.02 M sodium diethyldithiocarbonate, and 5 X 2 min DMF.[12, 16] 5-TAMRA-SE (2.5 mg, 0.005 mmol, 0.9 equiv in 1 mL DMF) was then attached to the free amine in the protected peptide resin (31 mg, 0.0056 mmol) using the same method as described for the synthesis of **1** (Scheme A2.1).

Peptide **4** was synthesized by SPPS on the Fmoc-Thr(*t*Bu)-Wang resin (0.50 mmol/g) according to procedure described for the synthesis of **1**.

Scheme A2.1: Synthesis of [Lys(5-TAMRA)^2]Dyn A, **2**

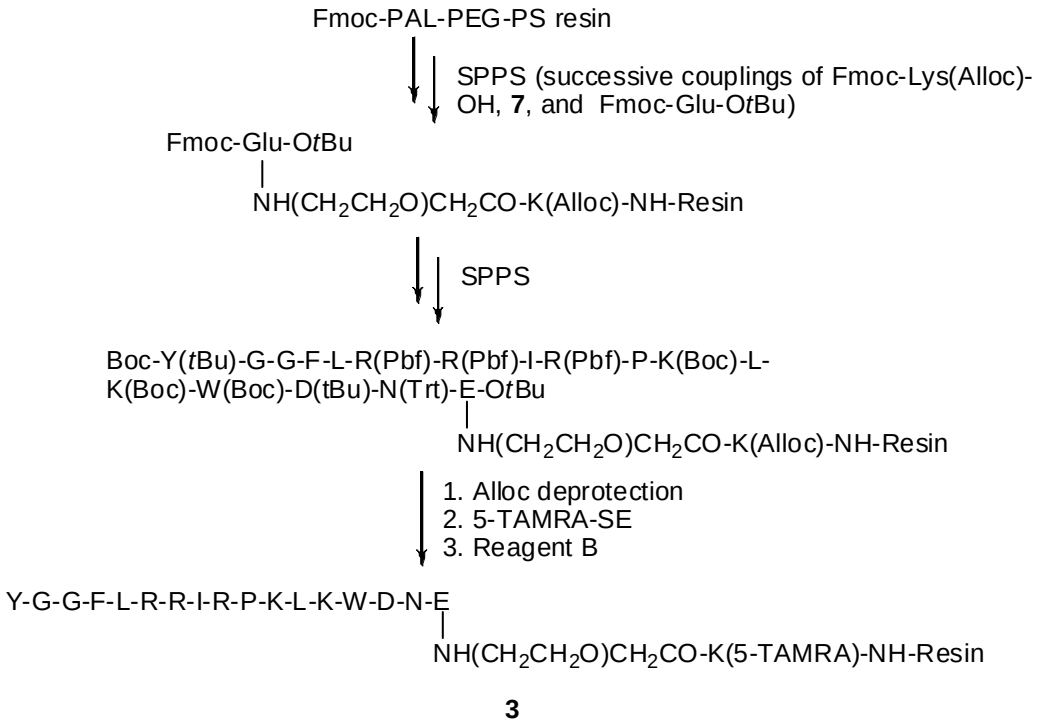


A2.3.3 Synthesis of 3 and 5

The Fmoc-PAL-PEG-PS resin was used to synthesize **3** (Scheme A2.2). Fmoc-Lys(Alloc)-OH, Fmoc-protected linker **7**, and Fmoc-Glu-O*t*Bu (2 equiv coupling for 2 h) were first successively attached to the resin by standard coupling reactions, followed by the coupling of the remaining amino acids until the whole protected linear peptide was assembled. The Alloc group was then deprotected using $Pd(PPh_3)_4$-$PhSiH_3$, followed by

attachment of the fluorescent tag 5-TAMRA as described above for the synthesis of compound **2**. The retention times for the non-fluorescent precursor (after Alloc deprotection) and the fluorescent peptide were 20.70 and 25.18 min, respectively, under the standard HPLC conditions (System 1, see section A2.3.6 for details). The yield (based on 5-TAMRA) for this reaction was 78% based on HPLC.

Scheme A2.2: Synthesis of the Dyn A analog **3** with the fluorescent group attached at the C-terminus



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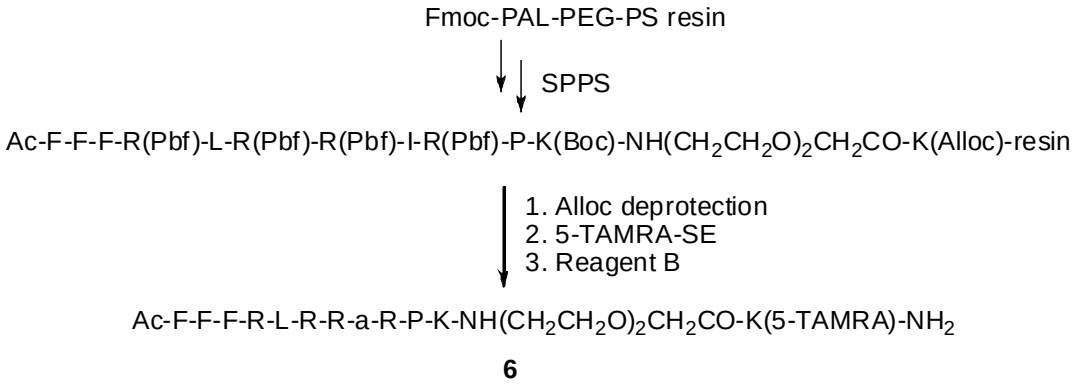
The fluorescent Dyn B analog **5** was synthesized by the same method as described for **3**. The retention times for the non-fluorescent precursor (after Alloc deprotection) and the fluorescent compound were 18.04 and 24.31 min, respectively, under the standard

HPLC conditions (System 1, see section A2.3.6 for details). The yield (based on 5-TAMRA) for this reaction was 77% based on HPLC.

A2.3.4 Synthesis of 6

The fluorescent peptide **6** was synthesized by SPPS on the Fmoc-PAL-PEG-PS resin (0.19 mmol/g) according to standard procedures (Scheme A2.3). Fmoc-Lys(Alloc)-OH (2 equiv) was first attached to PAL-PEG-PS resin, followed by the PEG-like linker **7** (2 equiv) and the remaining amino acids until the whole peptide was assembled. The N-terminal Fmoc group was then deprotected and the amine acetylated with excess amount (20 equiv) of acetic anhydride in DMF for half an hour. The Alloc group was then deprotected using $Pd(PPh_3)_4$ and $PhSiH_3$, as described for the synthesis of **2** above. The fluorescent tag was then attached to the peptide by reaction of 5-TAMRA-SE (2.5 mg, 0.005 mmol, 0.9 equiv) in DMF (1 mL) with the free amine. The reaction was allowed to run for 2 days and the completion of reaction was monitored by cleavage and HPLC analysis of an aliquot. The retention times for the non-fluorescent precursor (after Alloc deprotection) and the fluorescent compound were 22.20 and 27.38 min, respectively, under the standard HPLC conditions (System 1, see section A2.3.6 for details). The yield (based on 5-TAMRA) for this reaction was 92% based on HPLC.

Scheme A2.3: Synthesis of the arodyn analog **6** with the fluorescent group attached at the C-terminus



A2.3.5 Peptide cleavage, purification, and analysis

The fluorescent peptides were cleaved from the resin using reagent B (85% TFA, 5% phenol, 5% water, and 2% triisopropylsilane) at room temperature for 2 h.[17] The crude peptides were purified by preparative reversed-phase HPLC using a linear gradient of 15-50% MeCN containing 0.1% TFA over 35 min, at a flow rate of 20 mL/min. The purification was monitored by UV absorbance at 214 nm.

The purity of the final peptides was verified using analytical HPLC in two different solvent systems. In system 1, a linear gradient of 5-50% solvent B (solvent A, aqueous 0.1% TFA, and solvent B, MeCN containing 0.1% TFA) over 45 min, at a flow rate of 1 mL/min, was used with monitoring at 214 nm. In system 2, a linear gradient of 5-77% solvent B (solvent A, aqueous 0.1% TFA, and solvent B, MeOH containing 0.1% TFA) over 45 min, at a flow rate of 1 mL/min, was used with monitoring at 230 nm. Molecular weights of the compounds were determined by ESI-MS (Table A2.2).

A2.4 Bibliography

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Appendix 3. Design and Synthesis of Novel Peptide Antagonists for Mu Opioid Receptors Using a Reversed "Message-Address" Concept

*Note that the compound numbers used in this appendix are only applicable within this specific appendix.

A3.1 Introduction

The "message-address" concept for dynorphin (Dyn) A[1] has been successfully used in our laboratory to develop peptide antagonists for kappa (κ) opioid receptors.[2, 3] Arodyn (**1**) is a potent and highly selective antagonist at κ opioid receptors.[3] However, during the study of the structure-activity relationships (SAR), one analog [CH_3OCO-NMePhe[1],Nal(2')[3]]arodyn (**2**, Nal(2') = 2-naphthylalanine) exhibits much higher affinity for mu (μ) opioid receptors (K_i = 51.5 nM), and only about 2-fold selectivity for κ over μ opioid receptors (Table A3.1 and Chapter 4). The "message-address" concept suggests that the C-terminal "address" sequence of Dyn A/arodyn is important for affinity at κ opioid receptors. Therefore, deletion of the "address" sequence would be expected to dramatically decrease affinity for κ opioid receptors. If the μ opioid receptor affinity can be preserved, we can switch the selectivity from κ to μ opioid receptors. The truncation of the C-terminal sequence also makes the peptides much smaller. These truncated peptides might represent a novel pharmacophore for a μ opioid receptor antagonist.

The acetyl analog [NMePhe[1],Nal(2')[3]]arodyn (**3**) also shows similar binding affinity for μ opioid receptors (Table A3.1).[4] These two compounds (**2** and **3**) are exceptions since most of the arodyn analogs show very weak affinity for μ opioid receptors (i.e. K_i in the μM range). Subtle changes in the "message" residues of **2** or **3** decrease the μ opioid receptor binding affinity dramatically (Table A3.1). For example, the deletion of the N-methyl group of NMePhe[1] or substitution of NMePhe[1] by the bulky aromatic amino acid Nal(2') result in compounds **4** and **5** with about 6.8- and 3.7-fold lower affinity for μ opioid receptors, respectively, compared to **3** (Table A3.1).[4] Similarly, substitution of Nal(2') in position 3 with other aromatic (**6-8**) or non-aromatic

amino acids (**9**) also results in dramatically decreased affinity for μ opioid receptors (Table A3.1). These results suggest that residues NMePhe[1] and Nal(2')[3] are important for the binding affinity of **2** and **3** for μ opioid receptors. Therefore, we hypothesized that the N-terminus of **2** (at least residues 1 and 3) is necessary for affinity at μ opioid receptors.

Table A3.1: Mu opioid receptor affinities of arodyn (**1**) and its analogs

X-R_1-Phe-R_3-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2

Compound	X-R_1	R_3	K_i (μ)
1 (arodyn)[a]	Ac-Phe	Phe	1750 ± 130 (3)
2	CH_3OCO-NMePhe	Nal(2')	51.5 ± 13 (4)
3[b]	Ac-NMePhe	Nal(2')	158 ± 50 (3)
4	Ac-Phe	Nal(2')	1080 ± 10 (2)
5[b]	Ac-Nal(2')	Nal(2')	587 ± 51 (3)
6	CH_3OCO-NMePhe	Trp	1280 ± 280 (3)
7	CH_3OCO-NMePhe	Tyr	1300 ± 340 (4)
8	CH_3OCO-NMePhe	Phe	1750 ± 97 (3)
9	CH_3OCO-NMePhe	Cha	860 ± 350 (3)

[a]From reference 3; [b]From reference 4.

Compound **3**, an [NMePhe[1]]arodyn analog, is prone to a side reaction involving deletion of Ac-NMePhe from the peptide during acidic cleavage from the resin (see Chapter 3). Its analog where the acetyl group is replaced with the methoxylcarbonyl group (compound **2**) is stable under the same conditions. Therefore, compound **2** is used as the parent compound for the truncation study.

A3.2 Results, discussion, and conclusions

The main purpose of this truncation study is to determine the minimum residues for μ opioid receptor binding and delete as many residues important for κ opioid receptor binding as possible to convert the slightly κ opioid receptor selective analog **2** to a small

μ opioid receptor selective antagonist. Peptides with 3 to 7 amino acid residues (**10-14**, Figure A3.1) from the N-terminus of the parent compound were synthesized using the Fmoc-PAL-PEG-PS resin according to standard solid-phase peptide synthetic procedures (please refer to previous chapters for the detailed synthetic procedures).

Figure A3.1: Structures of **2** and its truncated analogs **10-14**

2: CH_3OCO-NMePhe-Phe-Nal(2')-Arg-Leu-Arg-Arg-D-Ala-Arg-Pro-Lys-NH_2

10: CH_3OCO-NMePhe-Phe-Nal(2')-NH_2

11: CH_3OCO-NMePhe-Phe-Nal(2')-Arg-NH_2

12: CH_3OCO-NMePhe-Phe-Nal(2')-Arg-Leu-NH_2

13: CH_3OCO-NMePhe-Phe-Nal(2')-Arg-Leu-Arg-NH_2

14: CH_3OCO-NMePhe-Phe-Nal(2')-Arg-Leu-Arg-Arg-NH_2

These peptides were purified by preparative HPLC; the final purity of the purified peptides was greater than 95% as determined by two analytical HPLC systems. ESI-MS was used to verify the desired molecular weights for these peptides (Table A3.2).

Table A3.2: Analytical data of the purified peptides

Peptides	HPLC (t_R (min)/% Purity)		ESI-MS (m/z)	
	System 1[a]	System 2[b]	Calculated	Observed
10	37.23/100	36.14/98.3	$[2M+Na]^+$=1183.5 $[M+Na]^+$=603.3 $[M+K]^+$=619.3	$[2M+Na]^+$=1183.5 $[M+Na]^+$=603.3 $[M+K]^+$=619.3
11	35.49/97.1	32.32/95.9	$[M+H]^+$=734.7	$[M+H]^+$=734.7
12	38.88/98.5	38.91/97.3	$[M+H]^+$=850.5	$[M+H]^+$=850.4
13	34.32/100	37.66/98.0	$[M+2H]^{2+}$=503.8 $[M+TFA+H]^+$=1120.6	$[M+2H]^{2+}$=503.7 $[M+TFA+H]^+$=1120.5
14	34.57/100	33.47/98.6	$[M+3H]^{3+}$=388.2 $[M+TFA+2H]^{2+}$=638.8	$[M+3H]^{3+}$=388.2 $[M+TFA+2H]^{2+}$=638.8

[a]System 1: Solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in acetonitrile, wavelength 214 nm. The gradient was 5-50% solvent B over 45 min at 1 mL/min. [b]System 2: Solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in methanol, wavelength 230 nm. The gradient was 25-70% solvent B over 45 min at 1 mL/min.

These truncated peptides are currently undergoing pharmacological evaluations for their opioid receptor affinity and selectivity as well as efficacy. We expect that some of these truncated analogs will retain reasonable affinity for μ receptors while exhibiting decreased affinity for κ receptors, therefore resulting in compounds that may be selective for μ opioid receptors. Further modifications will be based on the pharmacological activities of these truncated compounds. The analogs with the highest potency and/or selectivity at μ opioid receptors will be chosen as parent compounds for further modifications.

A3.3 Experimental section

The general synthesis, purification and analysis of these peptides have been described in previous chapters. Since these compounds are relatively hydrophobic, diethyl ether was not used to extract adducts of the scavengers with the side-chain protecting groups (e.g. Pbf group in the side chain of Arg) following cleavage. The TFA

was removed and these compounds were then purified by prep-HPLC. These compounds were purified using a linear gradient of 15-60% MeCN containing 0.1% TFA over 45 min, at a flow rate of 20 mL/min. The purification was monitored by UV absorbance at 214 nm.

A3.4 Bibliography

1. Chavkin, C.; Goldstein, A. Specific Receptor for the Opioid Peptide Dynorphin: Structure-Activity Relationships. *Proc. Natl. Acad. Sci. U.S.A.* **1981,** 78, 6543-6547.
2. Wan, Q.; Murray, T. F.; Aldrich, J. V. A Novel Acetylated Analogue of Dynorphin A-(1-11) Amide as a κ-Opioid Receptor Antagonist. *J. Med. Chem.* **1999,** 42, 3011-3013.
3. Bennett, M. A.; Murray, T. F.; Aldrich, J. V. Identification of Arodyn, a Novel Acetylated Dynorphin A-(1-11) Analogue, as a κ Opioid Receptor Antagonist. *J. Med. Chem.* **2002,** 45, 5617-5619.
4. Bennett, M. A. Synthesis and Evaluation of Opioid Activity of Novel Dynorphin A-(1-11) Analogues Using Classical and Combinatorial Library Approaches. Ph. D. Thesis, Department of Pharmaceutical Sciences, The University of Maryland Baltimore, 2003.

Appendix 4. ^{1}H-NMR Spectra of Arodyn Analogs Cyclized by RCM

*Note that the compound numbers used in this appendix are the same as those used in Chapter 7.

Figure A4.1: ^{1}H-NMR spectrum of **2**-*cis*.

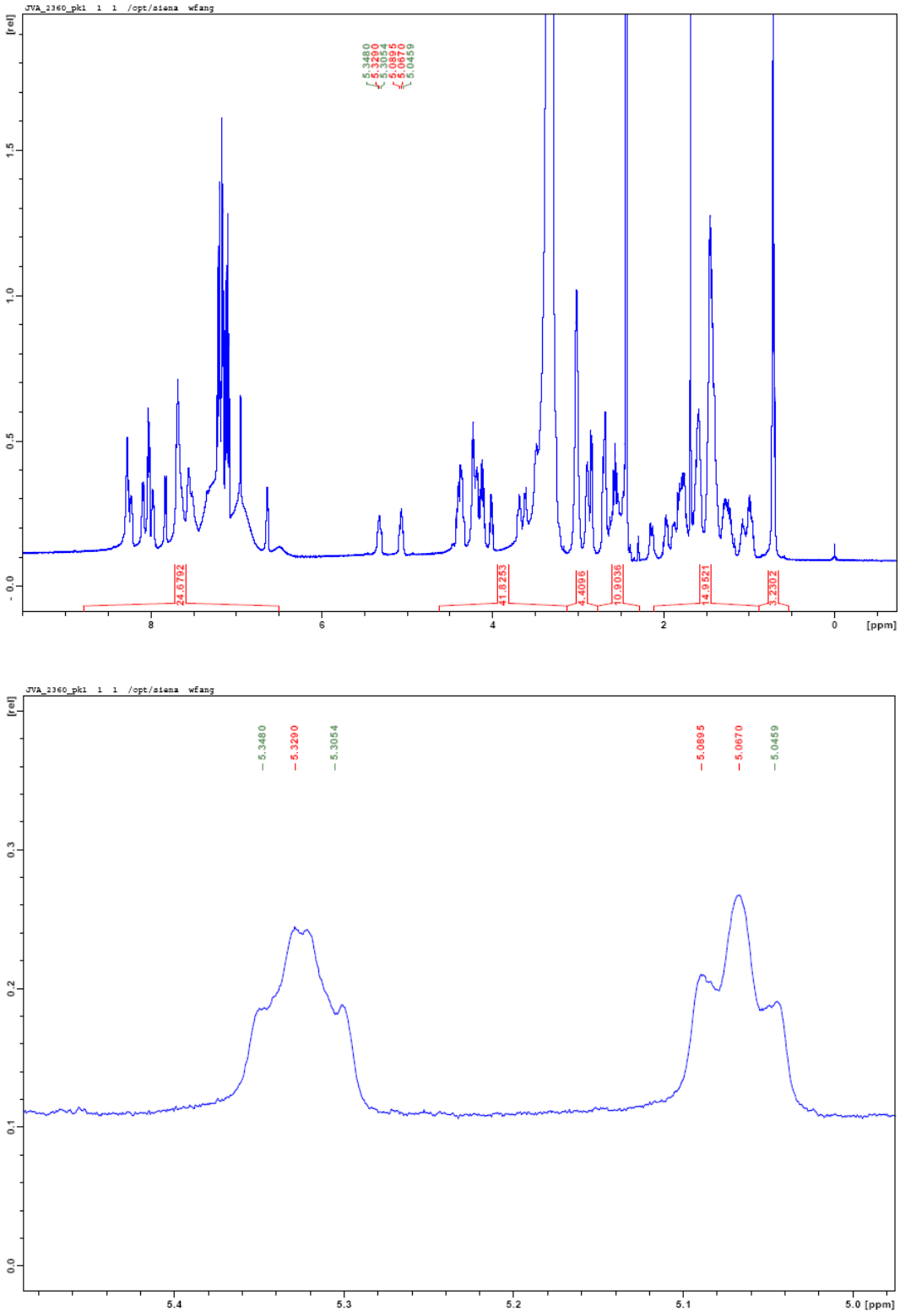


Figure A4.2: ^{1}H-NMR spectrum of **2**-*trans*.

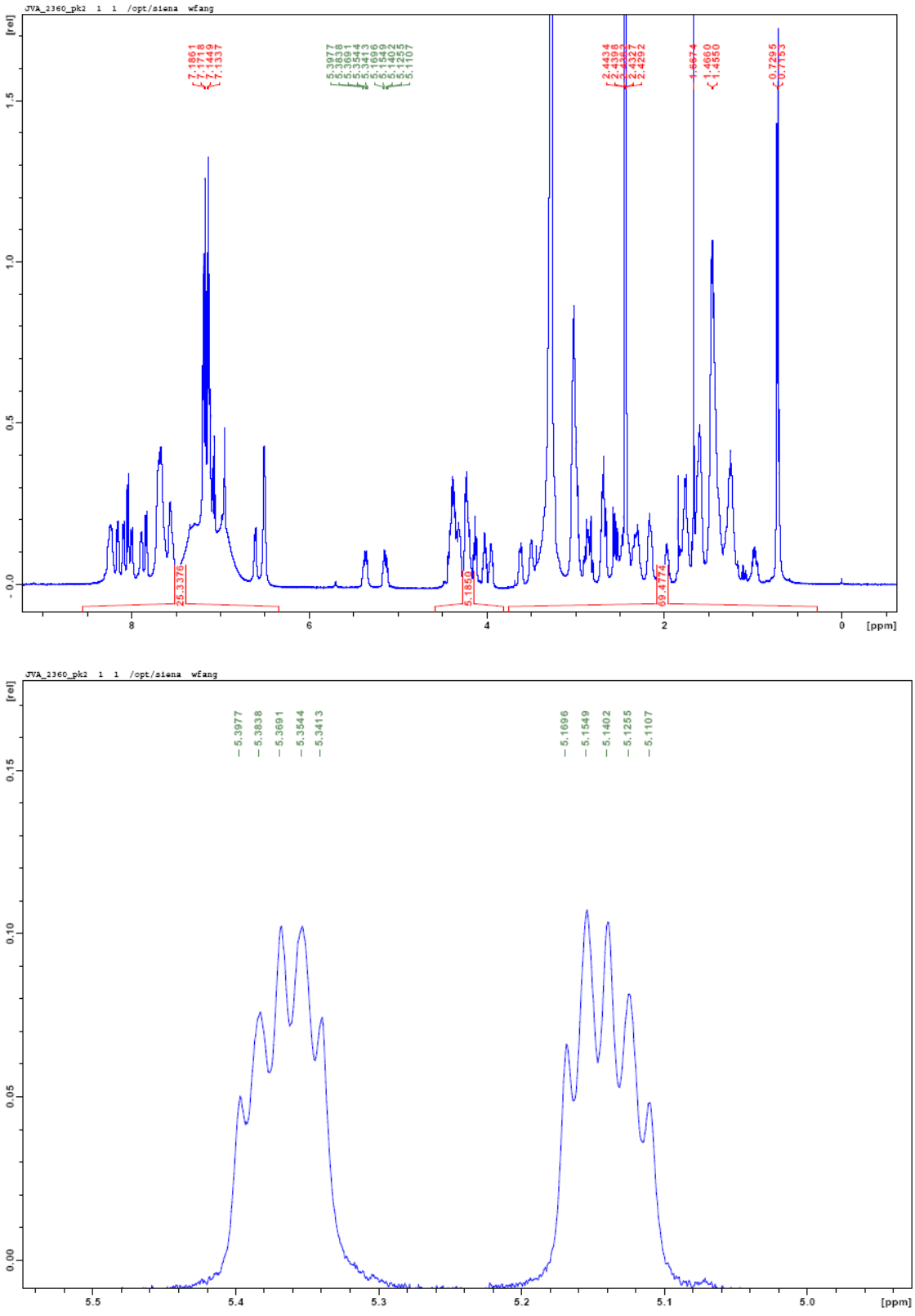


Figure A4.3: ^{1}H-NMR spectrum of **3**-*cis*.

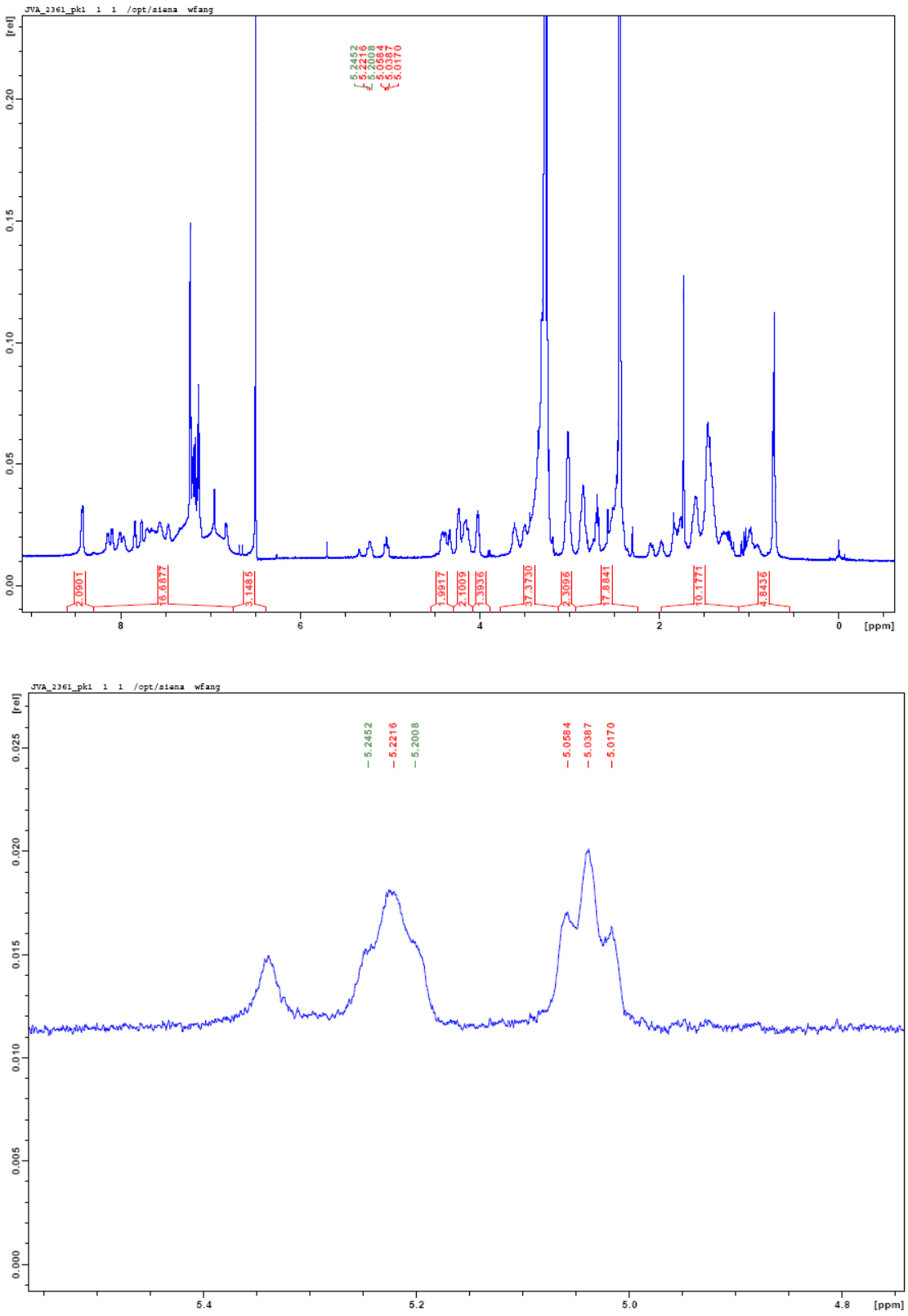


Figure A4.4: ^{1}H-NMR spectrum of **3**-*trans*.

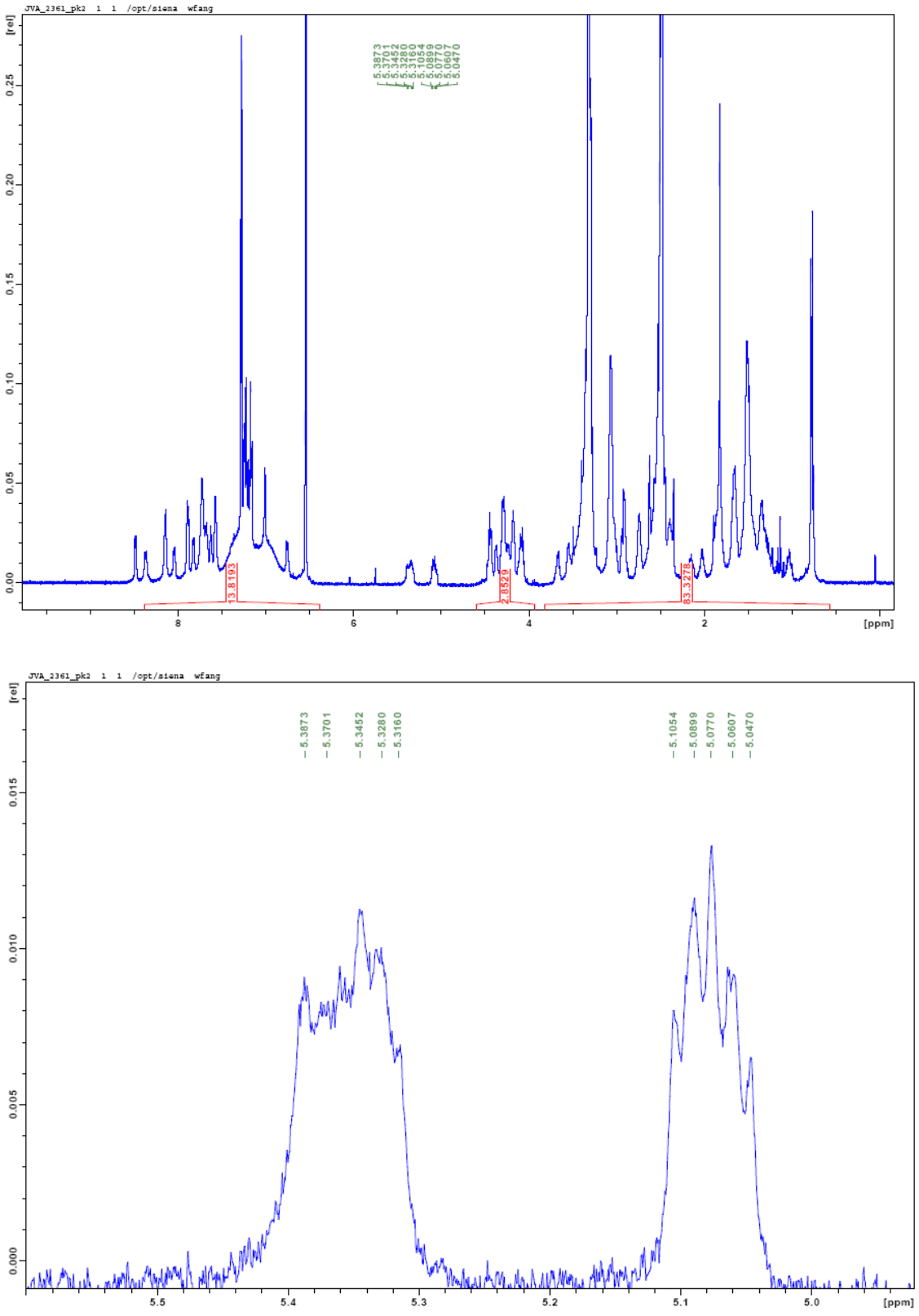


Figure A4.5: ^{1}H-NMR spectrum of **4**-*trans*.

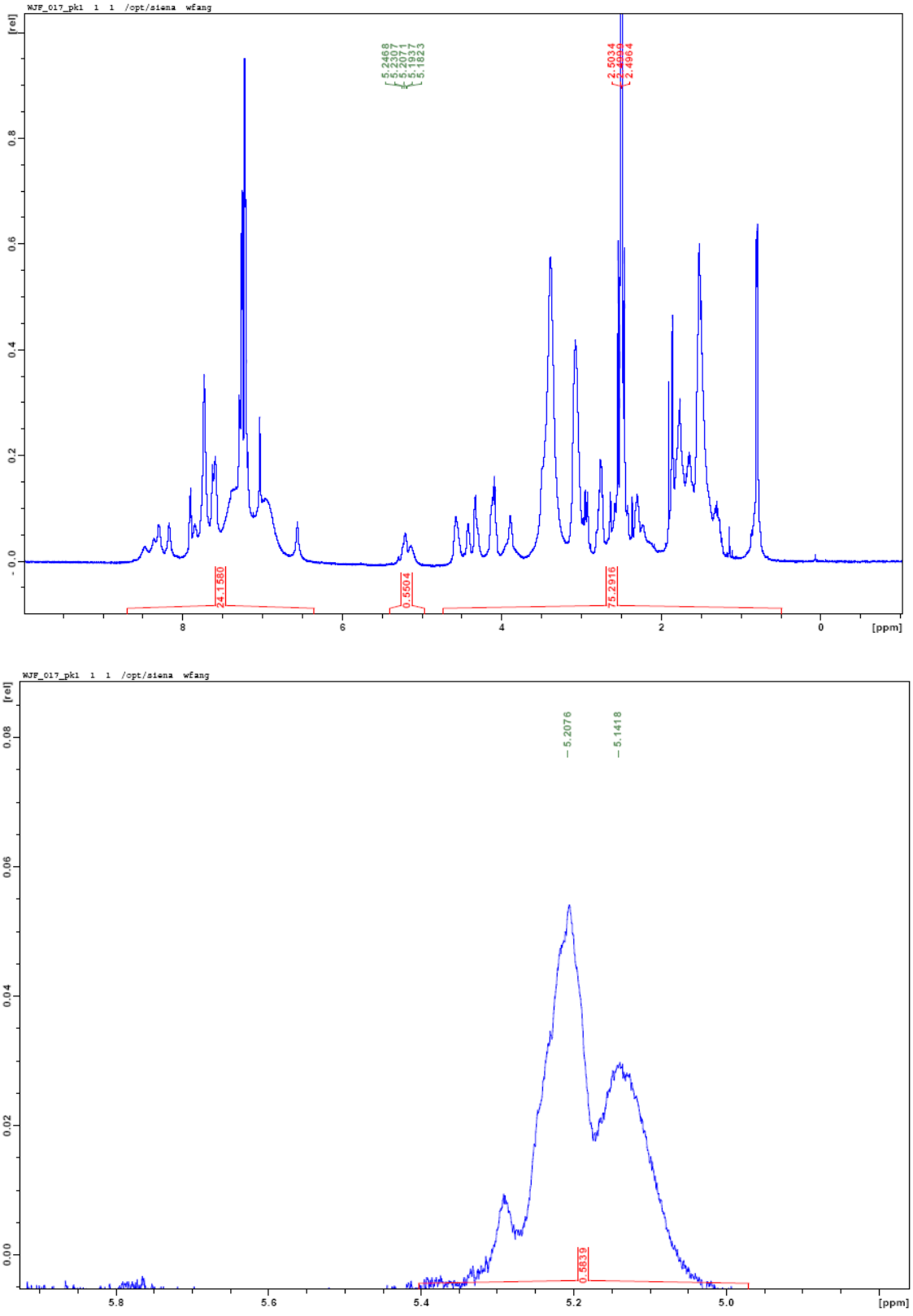


Figure A4.6: ^{1}H-NMR spectrum of **5**-*trans*.

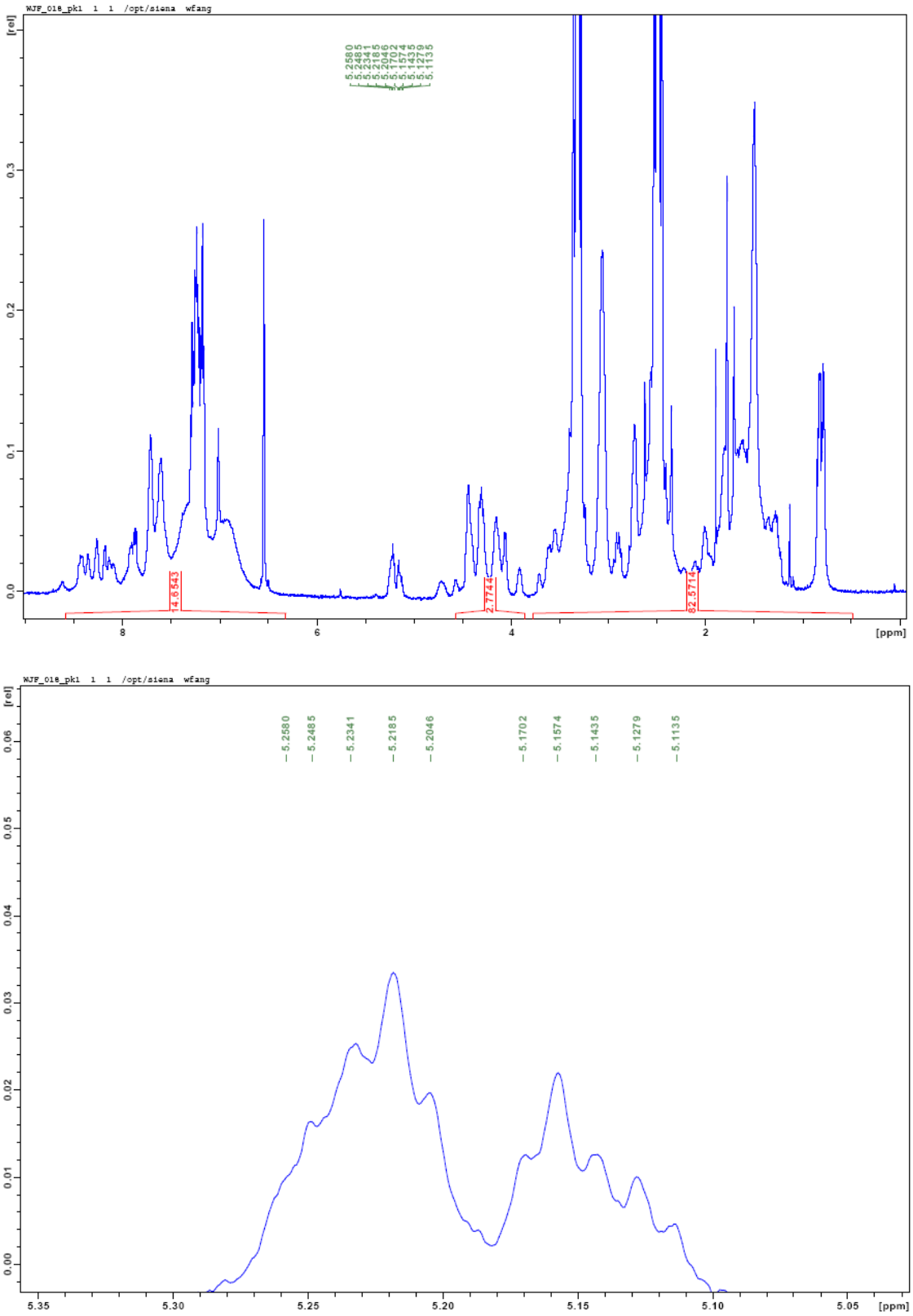


Figure A4.7: ^{1}H-NMR spectrum of **6**-*cis*.

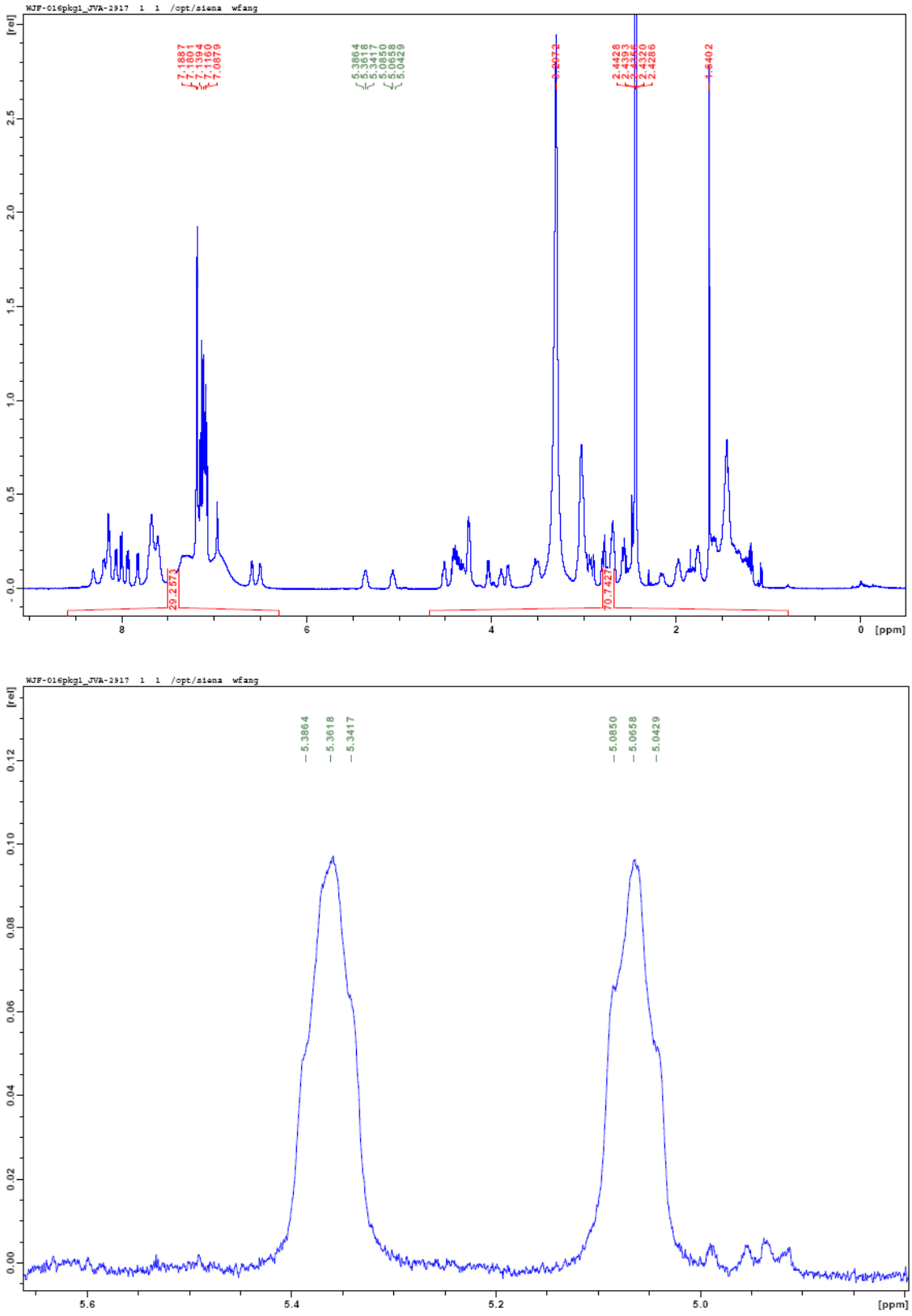


Figure A4.8: ^{1}H-NMR spectrum of **6**-*trans*.

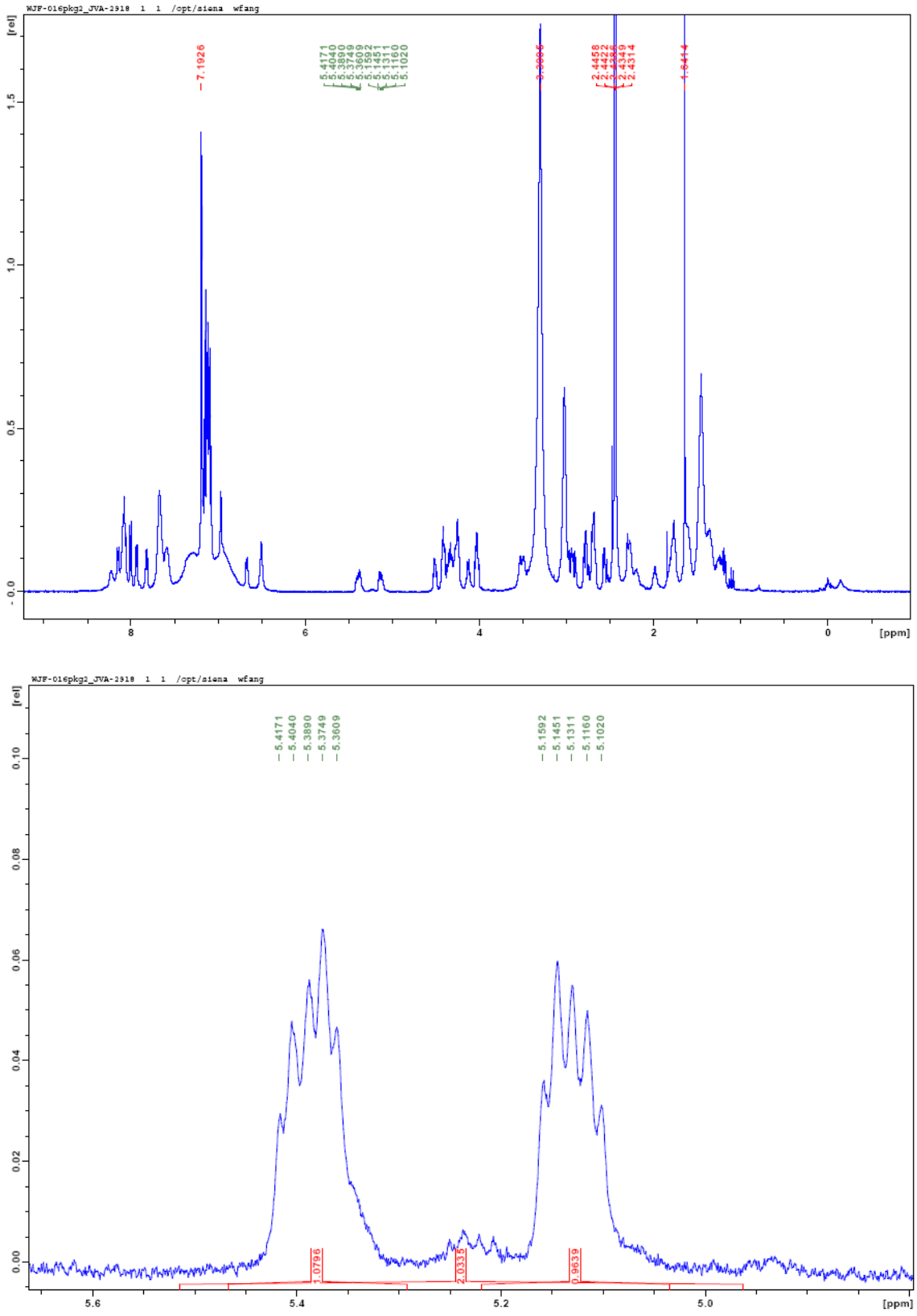


Figure A4.9: [1]H-NMR spectrum of **16** (*trans)*. Bottom is the spectrum for the two vinyl protons after decoupling of adjacent methylene protons at 2.36 ppm.

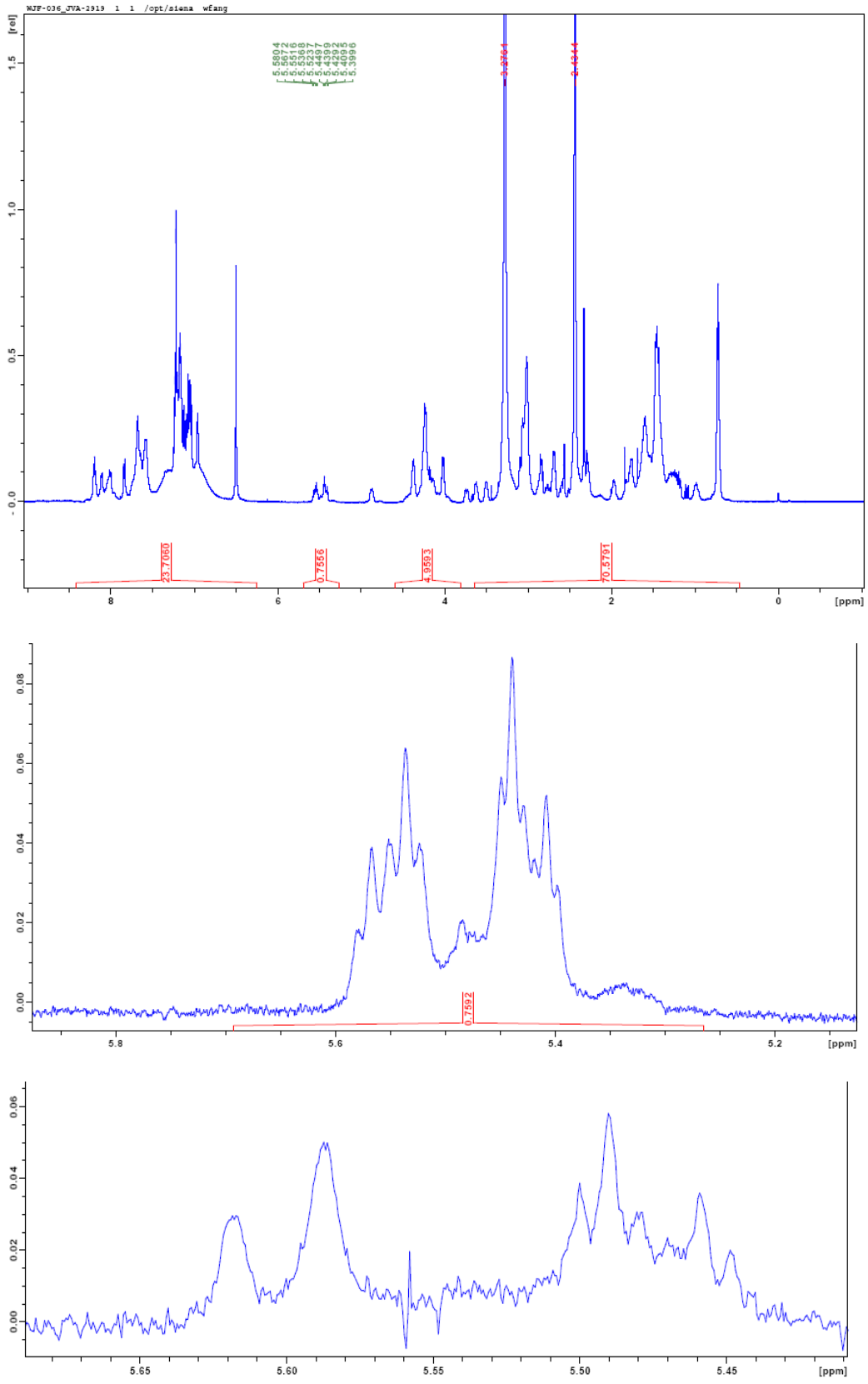


Figure A4.10: ^{1}H-NMR spectrum of **21** (*trans)*. Bottom is the spectrum for the two vinyl protons after decoupling of adjacent methylene protons at 4.61 ppm.

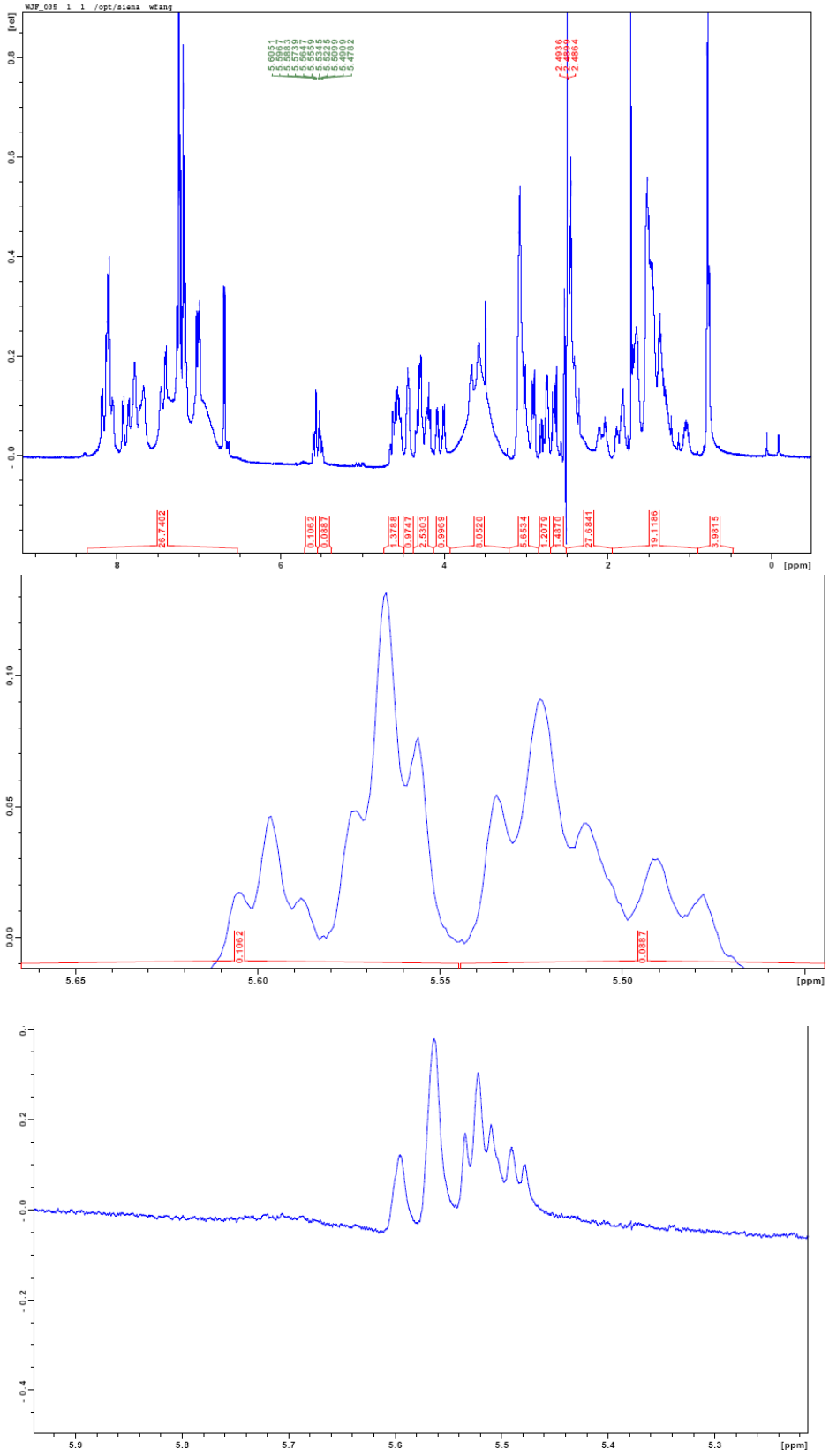


Figure A4.11: [1]H-NMR spectrum of **22** (*trans)*. Bottom is the spectrum for the two vinyl protons after decoupling of adjacent methylene protons at 4.65 ppm.

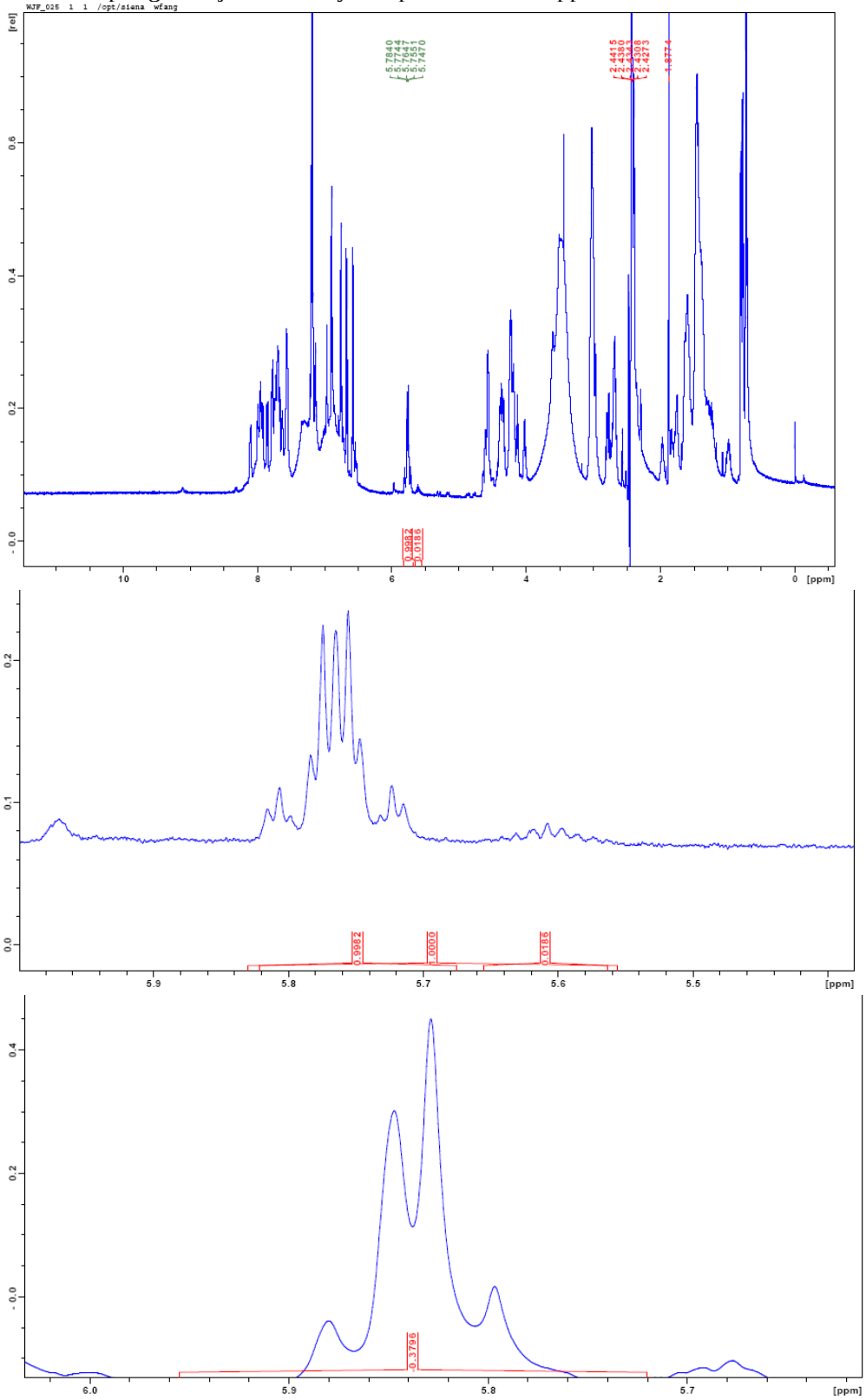
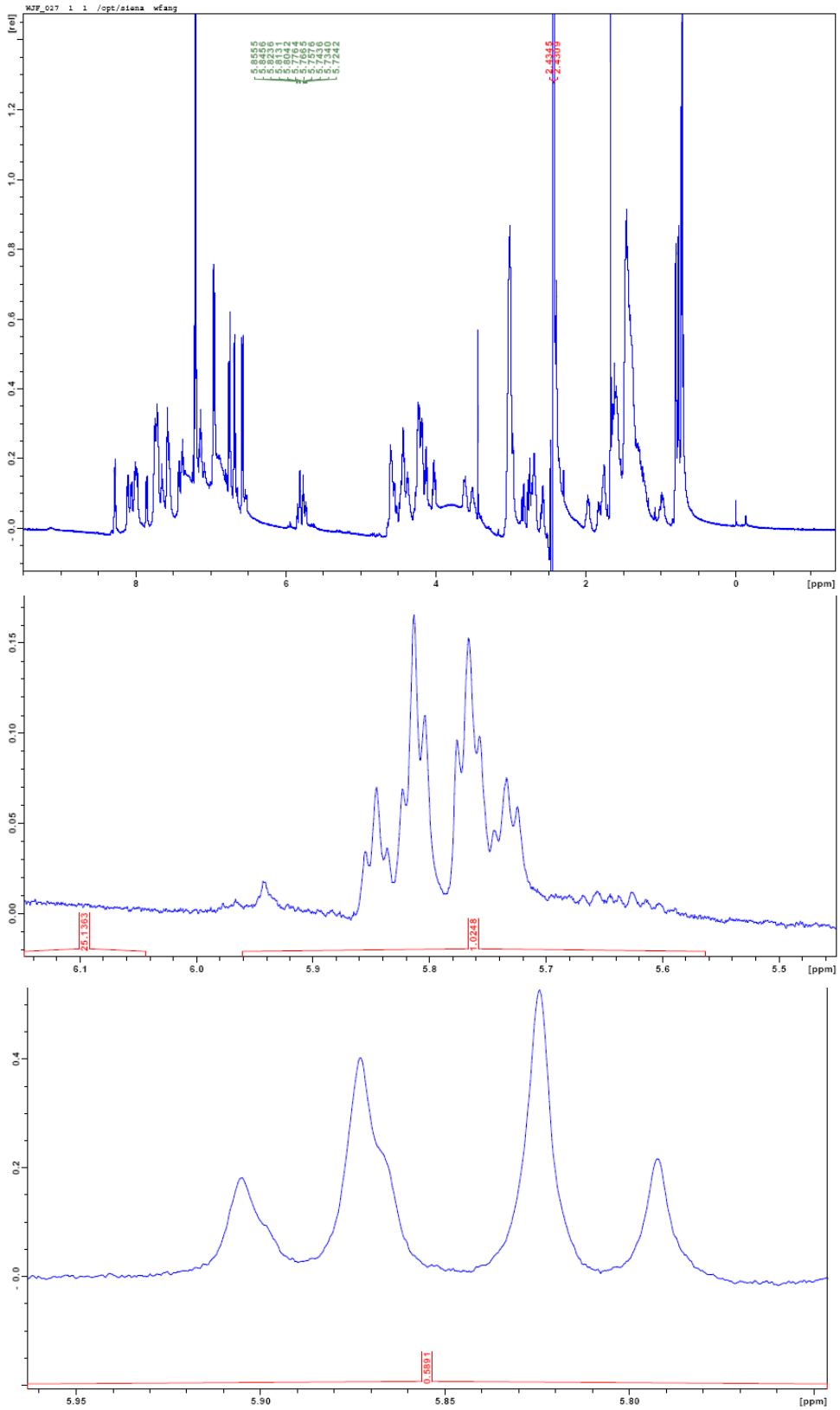


Figure A4.12: ^{1}H-NMR spectrum of **23** (*trans)*. Bottom is the spectrum for the two vinyl protons after decoupling of adjacent methylene protons at 4.61 and 4.66 ppm.



Appendix 5. ^{1}H-NMR Spectra of Dyn A Analogs Cyclized by RCM

*Note that the compound numbers used in this appendix are the same as those used in Chapter 8.

Figure A5.1: ^{1}H-NMR spectrum of **1** (*cis*).

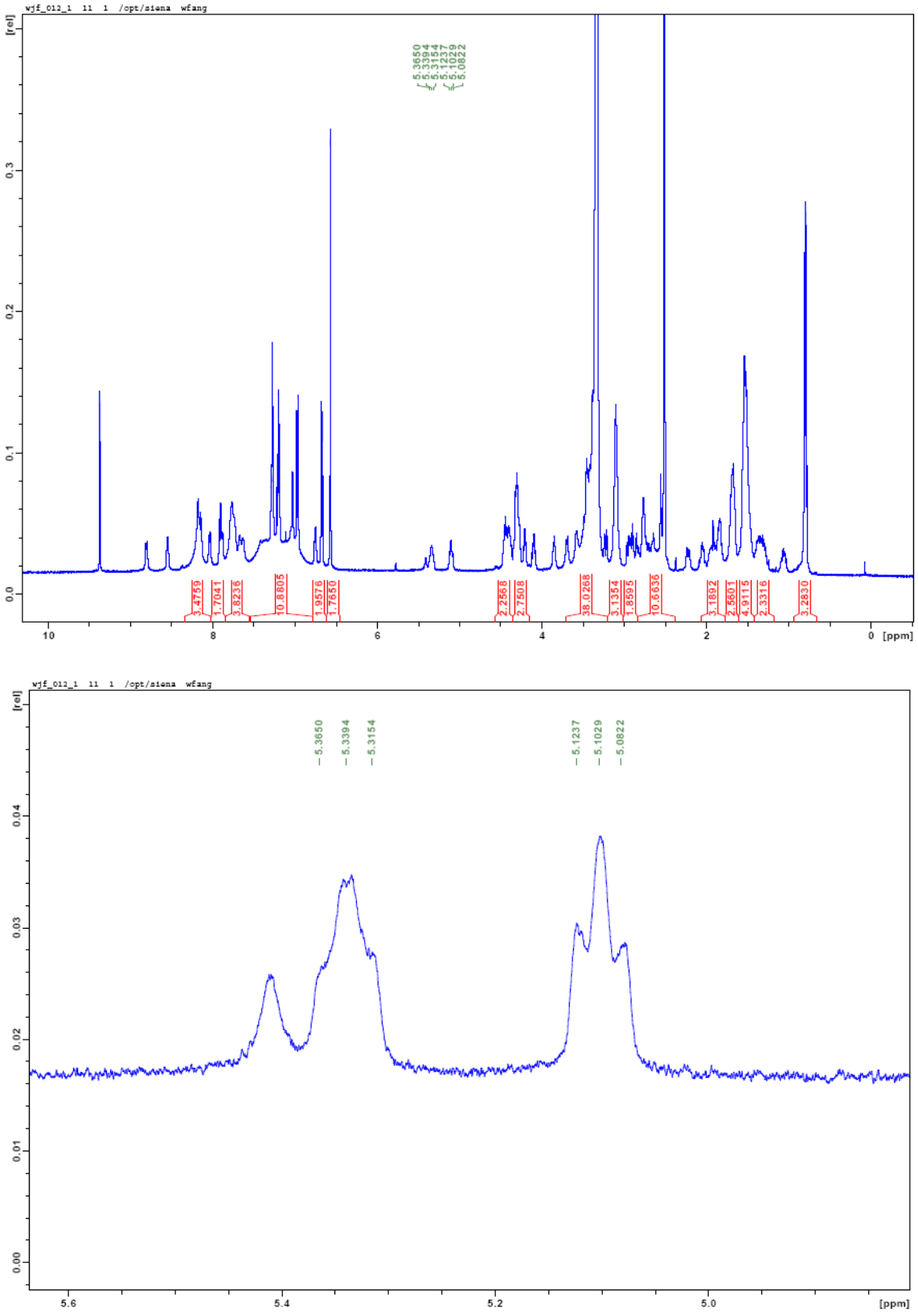


Figure A5.2: ^{1}H-NMR spectrum of **2** (*trans*).

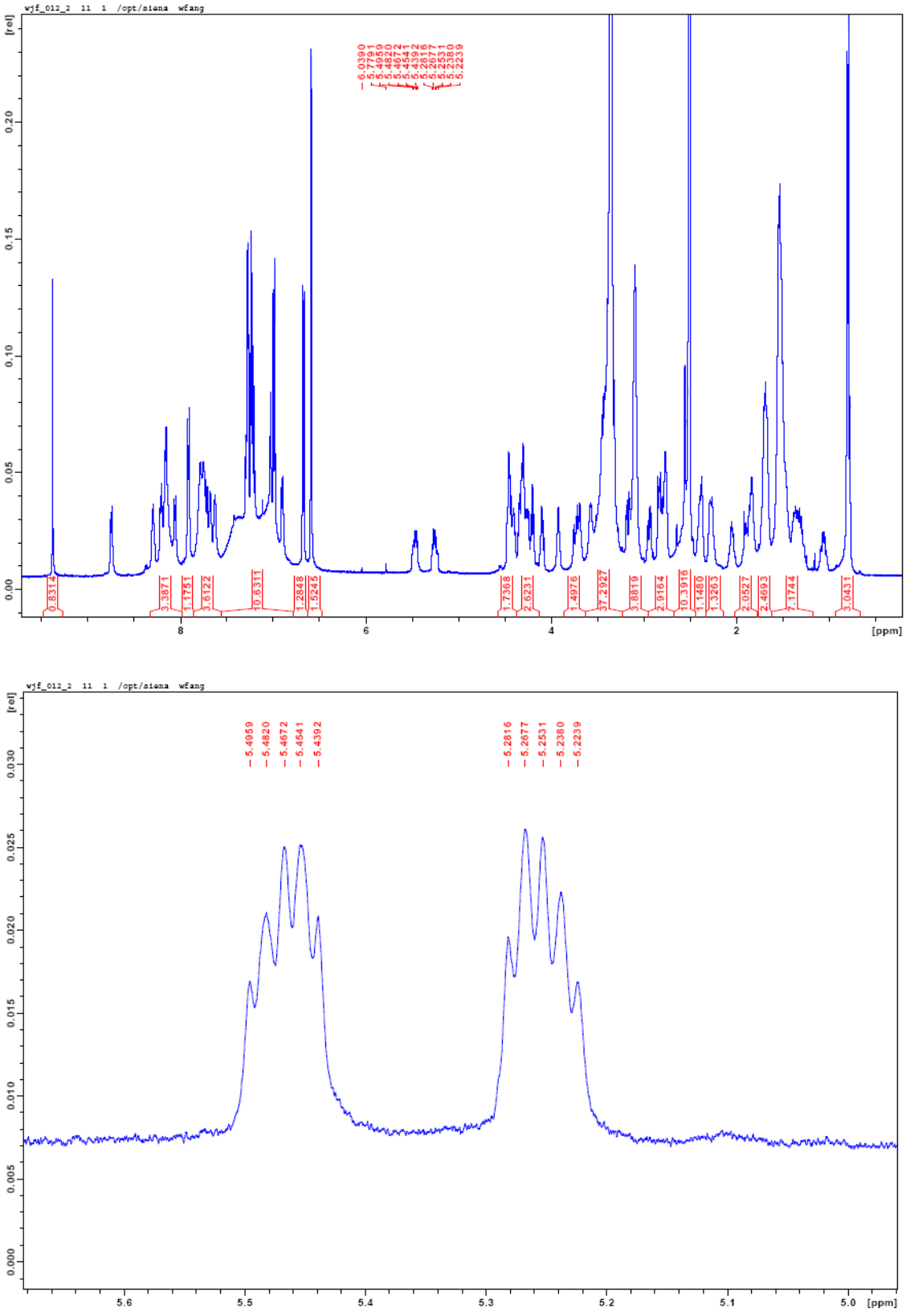


Figure A5.3: ^{1}H-NMR spectrum of **3** (*cis*).

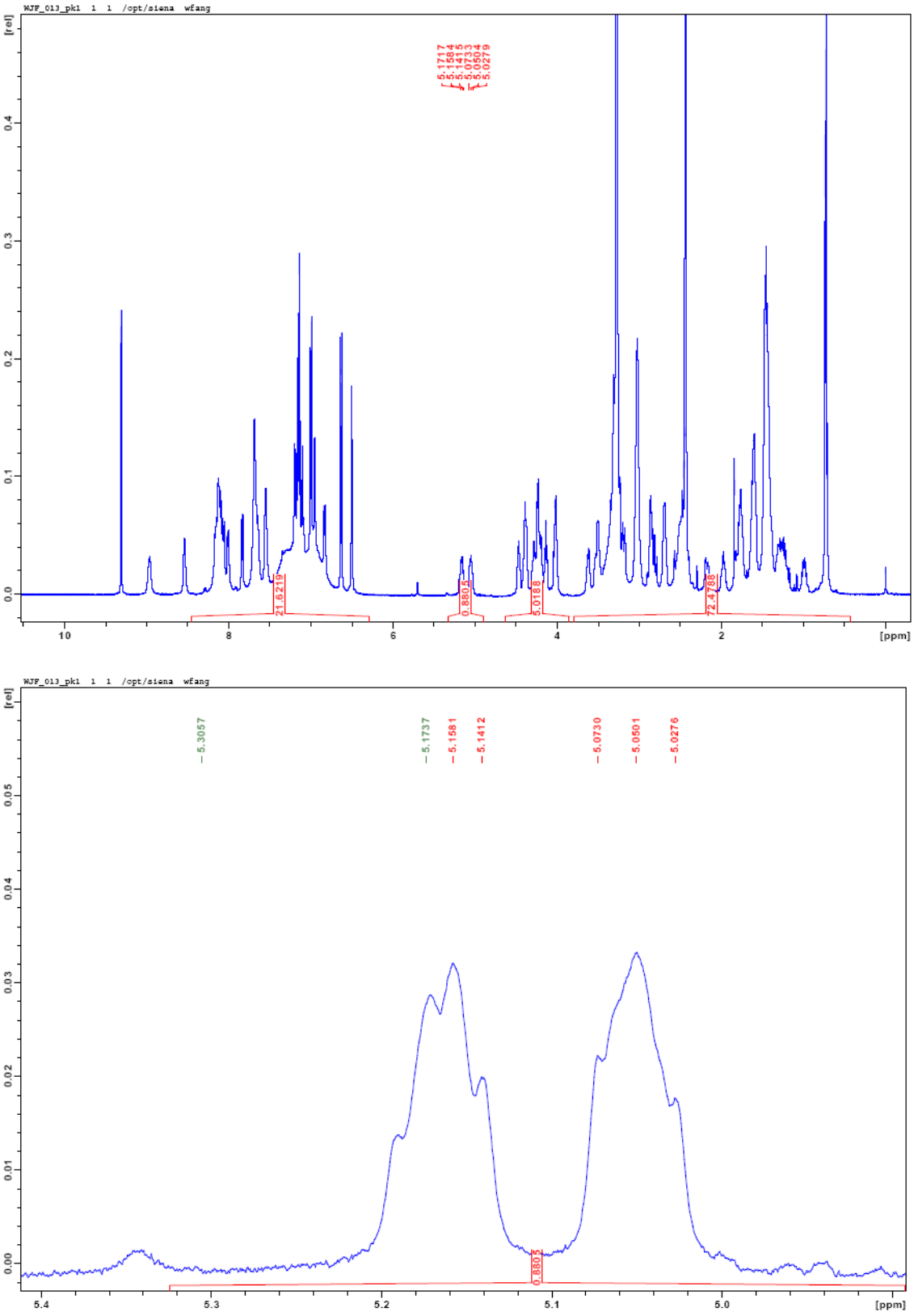


Figure A5.4: ^{1}H-NMR spectrum of **4** (*trans*).

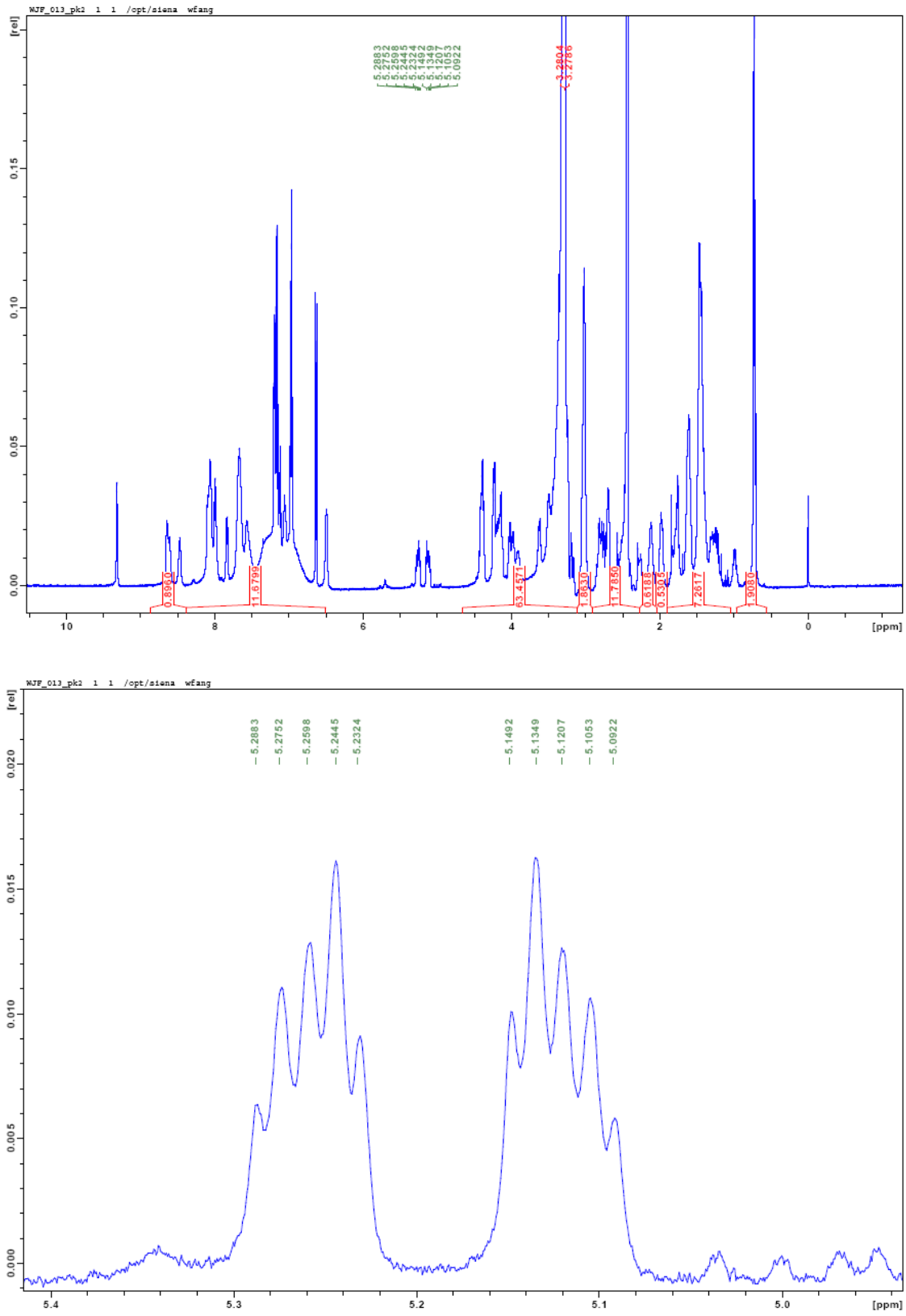


Figure A5.5: ^{1}H-NMR spectrum of **5** (*cis*).

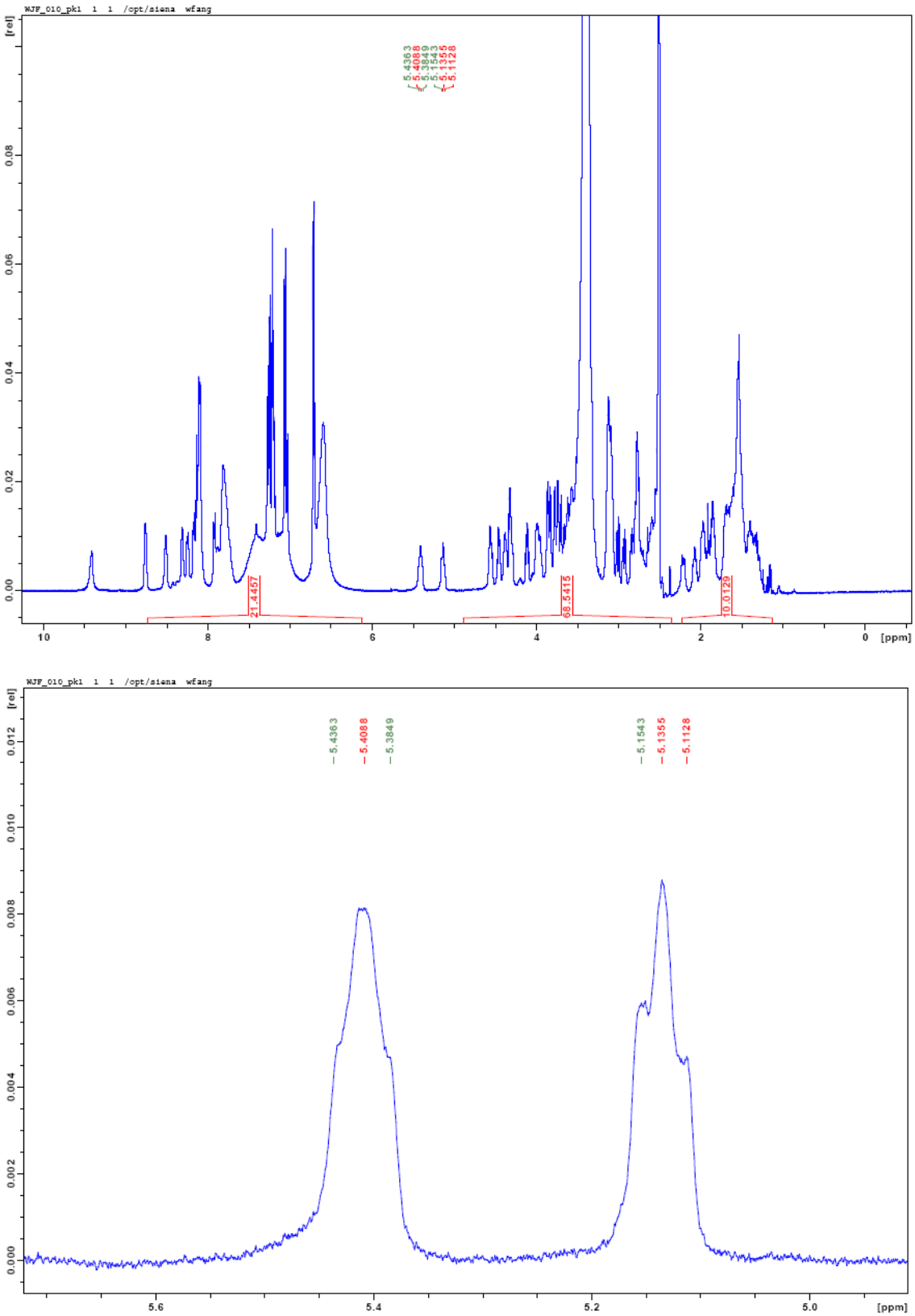


Figure A5.6: ^{1}H-NMR spectrum of **6** (*trans*).